THE ROLE OF CHFR AND UBC13 IN MITOSIS

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LIST OF ABBREVIATIONS

EGFP = Enhanced Green fluorescent Protein

siRNA = short interfering RNA

Ubc13 = ubiquitin-conjugating enzyme 13

Chfr = checkpoint protein with fork-head and RING finger domains

Uev1a = ubiquitin conjugating-enzyme variant

Mms2 = methyl methanesulfonate sensitive 2

CHFR = human gene, written in capital letters and italics

MMS2 = human gene, written in capital letter and italics

FHA = fork-head associated domain

RING = really interesting new gene domain

K48-linked poly-ubiquitin chain = a chain of many ubiquitin molecules, linked through amino acid lysine 48

K63-linked poly-ubiquitin chain = a chain of many ubiquitin molecules, linked through amino acid lysine 63

2xThymidine-1xRO3306 = double thymidine, single RO3306; a block using thymidine and the RO3306 drug to block cells at the G2/M stage

ICC = Immunocytochemistry

DIC = Differential interference contrast microscopy

HDAC = Histone Deacetylse

Plk1 = Polo-like kinase 1

EPI = Early prophase index

APC = Anaphase Promoting Complex

DMEM = Dulbecco's modified eagle medium

PBST = Phosphate buffered saline with Tween

IP =Immunoprecipitation

WT = Wild type

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ABSTRACT

The Chfr checkpoint is a point at which a cell checks whether it is safe to enter mitosis. Chfr is a protein that functions at this particular checkpoint to ensure safe entry into mitosis, but the molecular mechanism by which this protein functions is not entirely clear. The hypothesis in this thesis is that Ubc13, Chfr, and Uev1/Mms2 function together in mitosis during the Chfr checkpoint. The results were observed using immunocytochemistry, the mitotic shake off procedure, Western blot analysis, and coimmunoprecipitation. High Ubc13, Mms2, and Chfr-Ub levels at the interphase-early prophase transition indicate that these proteins function together at the Chfr checkpoint. Localization of Chfr to decondensed chromatin in interphase cells and to decondensing chromatin in telophase cells indicates a decondensing function for Chfr. Interaction between Chfr and Ubc13, Chfr and phosphorylated histone H3, as well as Ubc13 and phosphorylated histone H3 further indicates that these proteins may function together at the Chfr checkpoint, because phosphorylated histone H3 is a mitotic protein at that particular point in mitosis. Localization of Chfr, Ubc13, and Mms2 to the centrosomes indicates that they function together at these sites to target substrates important in centrosome maturation, separation, and spindle formation. Furthermore, there are two molecular states of Chfr: Chfr and Chfr-Ub. Chfr is predominant at late prophase, whereas, Chfr-Ub is predominant at interphase-early prophase. Chfr increases in level upon nocodazole exposure at late prophase to counteract the mitotic stress; and it also looses its ubiquitin signal upon passage into mitosis. High Ubc13 and Mms2 levels coincide with high Chfr-Ub levels at the

interphase-early prophase transition, indicating that they function together at the Chfr checkpoint. The ubiquitin signal could be either K-48-linked or K-63-linked in nature. We propose that the Chfr, Ubc13, and Mms2 protein complex could function through an auto-ubiquitination-decondensation-Chfr destruction-recondensation mechanism. Furthermore, Chfr could bind to pH3 and its auto-ubiquitin signal to serve as a bulky modification that hinders chromosome condensation.

CHAPTER 1: INTRODUCTION

During mitosis a cell must accurately divide itself and its two meters of genetic material into two daughter cells. The vulnerability of mitosis often manifests itself in the form of cancer. Mitotic inaccuracy threatens genetic instability, which could result not only in the demise of the cell containing that inaccuracy, but also in the demise of the organism as a whole.

It is likely for this reason that cells employ molecular machines, called "mitotic checkpoint proteins," to regulate their entry and progression through mitosis. If during mitosis cells encounter stress or detect a suspicious event that could pose a threat to accurate cell division, mitotic checkpoint proteins sense this threat, transduce a signal for help, and execute the appropriate response to that threat – giving a cell more time to cope with the stress.

Chfr is one such mitotic molecular machine (Privette et al., 2008a). The function of Chfr is presumed to stop mitotic entry under conditions that could compromise genetic stability of the cell. This protein is considered to be a tumor suppressor and is frequently down-regulated in human cancers by promoter hypermethylation, resulting in reduced Chfr protein levels, a high number of condensed chromosomes, and a high mitotic index (MI) following treatment with nocodazole (Earson and Petty, 2004; Privette et al., 2008a,b; Yu et al., 2005). Cells containing Chfr frequently have a lower number of condensed chromosomes and a lower mitotic index following treatment with nocodazole. Nocodazole disassembles microtubules and is a form of mitotic stress. *CHFR* is composed of at least three

functional domains: the N-terminal forkhead-associated (FHA) domain, the central RING finger domain, and the cysteine-rich C-terminal domain.

1.1 Chfr domain structure and functions.

1.1.1 The FHA domain of Chfr. The Chfr protein has three domains **(Figure 1-1).** Chfr has the ability to bind phosphorylated peptides through its FHA domain (Stavridi et al., 2002). A colony formation assay was used to show how expression of Chfr affects the growth of cells (Fukuda et al., 2008). Wild type Chfr, vector alone, or the Chfr FHA deletion mutant were expressed in HCT116 and RKO cells. The cells expressing the Chfr FHA deletion formed a fewer number of colonies/dish than did cells expressing the vector alone, and cells containing wild type Chfr had a substantially smaller number of colonies/dish than did cells expressing Chfr that lacked the FHA domain. The cells used for this experiment were not expected to contain endogenous Chfr (Fukuda et al., 2008). This indicates that, in the absence of the FHA domain, Chfr still contains some anti-proliferative activity, because this protein still reduces proliferation, but is not as efficient as wild type Chfr. In summary, the data demonstrate that the FHA domain affects the tumor suppressive quality of Chfr, perhaps because of its importance in substrate binding. Their results indicate that the FHA domain of Chfr plays a role in preventing mitotic progression, and when deleted partially reduces Chfr function, resulting in a higher number of mitotic cells. Absence of the FHA domain in Chfr may prevent proper binding of Chfr to its substrate. This could in turn interfere with proper ubiquitination of the

substrate, causing inefficient stress signaling, inefficient mitotic delay, and a higher likelihood of proliferation.

Using the mitotic index assay, one group showed that expression of the Chfr FHA deletion in SAOS2 cells, which are expected to contain the Chfr checkpoint and endogenous Chfr protein expression, actually results in an increased mitotic index in SAOS2 cells following nocodazole exposure (Scolnick and Halazonetis, 2000). This indicates that the Chfr FHA deletion protein in cells expressing Chfr may actually compete with the functional form of the protein for binding to substrate, preventing functional Chfr from binding to substrate and generating the expected signal for reducing mitotic index.

Expression of the Chfr FHA deletion mutant in control cells that either lacked Chfr expression (DLD-1) or already expressed a Chfr mutant (U2OS), had no effect on mitotic index following nocodazole exposure as expected. Only wild type Chfr expression had a clear effect on mitotic index in these cells. This indicates that the FHA domain of Chfr is important for its function (Scolnick and Halazonetis, 2000).

Using the DIC microscopy assay, Matsusaka and Pines showed using DIC microscopy that cells expressing wild type Chfr clearly return to interphase following treatment with a microtubule poison such as colcemid (Matsusaka and Pines, 2004). The wild type cells were then compared to cells that express a Chfr FHA deletion, and these particular cells were unable to return to interphase, progressing directly into mitosis (Matsusaka and Pines, 2004). This suggests that the FHA domain of Chfr is required for early prophase checkpoint function.

Figure 1-1. A diagram showing the Chfr domains. There is the fork-head associated (FHA) domain, the ring-finger (RF) domain, and the C-terminal cysteine rich (CR) domain.

By using a metaphase spread assay, it was shown that the FHA deletion in Chfr generates aneuploidy (Yu et al., 2005). This data indicates that the FHA domain of Chfr is important for preventing cells from progressing into mitosis with an abnormal number of chromosomes. Using the ubiquitination assay, it was demonstrated that when the FHA and RING finger domain of Chfr is present, Chfr auto-ubiquitinates itself lightly (Kang et al., 2002). The smaller constructs, which lack the FHA domain and contain only the RING finger domain, on the other hand, have more pronounced ubiquitin ligase activity. This indicates that the deletion of the FHA domain could increase the ability of Chfr to auto-ubiquitinate itself, improving the kinetics of the ubiquitination reaction (Kang et al., 2002).

1.1.2 The C-terminal cysteine-rich Chfr domain. Aurora A is one potential Chfr substrate (Yu et al., 2005). The C-terminal region of Chfr interacts with Aurora A, and is required for its ubiquitination. This group also showed that Chfr levels affect the levels of Aurora A in cells. Another potential substrate for Chfr is HDAC. Young et al. (2009) showed that the C-terminus of Chfr is required for its interaction with HDAC and is required for its ubiquitination. Increasing Chfr levels results in HDAC decrease.

Scolnick and Halazonetis (2000) detected a mutation in the Chfr protein that was naturally expressed in U2OS cells. The group generated cell lines stably expressing this mutation. The expression of this C-terminal mutant in DLD-1 cells, which are not expected to contain Chfr or the checkpoint, did not have an effect on their mitotic index, as compared to the vector control. Only wild type expression

had a prominent effect on mitotic index. This indicates that the C-terminal region of Chfr is important in reducing mitotic index (Scolnick and Halazonetis, 2000).

Chaturvedi et al. (2002) also showed that the C-terminal Chfr mutation was very similar to the vector in terms of MI, whereas wild type Chfr expression substantially reduced mitotic index. This indicates that the C-terminal domain is involved in preventing mitotic progression.

1.1.3 The RING domain of Chfr. The RING finger domain gives Chfr its E3 ubiquitin ligase activity, enabling the ligase to ubiquitinate itself, as well as other potential substrate proteins (Yu et al., 2005; Kang et al., 2002; Chaturvedi et al., 2002). The nature of the ubiquitination required to achieve the early prophase checkpoint is unclear. Some reports indicate that CHFR targets cell cycle promoting proteins such as Plk1 or Aurora A, for degradation through K48-linked poly-ubiquitin chains to delay mitotic entry in response to microtubule damage (Yu et al., 2005; Kang et al., 2002). Other reports support the idea that CHFR functions through K63-linked poly-ubiquitin chains possibly by targeting the p38 kinase pathway to achieve mitotic delay (Bothos et al., 2003; Matsusaka and Halazonetis, 2004). K63-linked poly-ubiquitin chains are not used for degradation, but rather to alter the activity of a particular target protein.

Wild type Chfr can ubiquitinate itself, but when the RING finger domain of Chfr is changed Chfr can no longer ubiquitinatinate itself (Kang et al., 2002). Thus the RING finger domain is necessary for Chfr auto-ubiquitination. This group also

showed that the presence or absence of the FHA domain influences the ability of the RING finger portion of Chfr to carry out the auto-ubiquitination process.

Wild type Chfr can ubiquitinate itself while the RING finger mutant of Chfr cannot ubiquitinate itself (Chaturvedi et al., 2002). Chfr lacking cells expressing vector alone or Chfr RING finger mutant cells have reduced survival than wild type Chfr containing cells. Expression of Chfr in this case improves viability of DLD-1 cells in taxol because the safety checkpoint is restored. Expression of the RING finger mutant or vector alone in these same cells decreases the viability because there is no safety checkpoint restored in this case.

Bothos et al. (2003) showed that Chfr mutants that have lost ubiquitin ligase activity could not reduce mitotic index. Cells expressing wild type Chfr, on the other hand, can reduce their own mitotic index.

According to Matsusaka and Pines (2004), U2OS cells expressing the RING finger mutant of Chfr progressed readily into mitosis, whereas cells expressing wild type Chfr did not progress into mitosis, remaining in an interphase-early prophase like state.

Kim et al. (2011) showed very clearly how the Chfr protein could function during the Chfr checkpoint. This group showed that when wild type Chfr is expressed in cells, Chfr levels decrease over time after release from the double thymidine block, while pH3 levels rise. This is an inverse relationship between Chfr and pH3 protein levels. This decrease in CHFR is probably due to a degradative auto-ubiquitin signal, which allows cells to enter mitosis. Chfr level decreases here over time because the Chfr protein becomes less and less necessary as cells progress

through mitosis. Kim et al. also points out here that wild type CHFR expression results in less pH3 protein expression in comparison to cells expressing no CHFR protein. This is in line with Chfr preventing cells from entering mitosis as Chfr protein levels go up, pH3 protein levels (mitotic indicators) go down. A Chfr mutant that contains ubiquitin ligase activity, but cannot auto-ubiquitinate itself for destruction (FLAG-Chfr K2A), on the other hand, remains stable over time in terms of protein level. Phospho-histone H3 levels do not increase over time in these cells, and remain much lower in comparison to wild type Chfr control cells, as Chfr level is high and prevents cells from entering mitosis (Kim et al., 2011). This indicates that auto-ubiquitination of Chfr is degradative in nature, and functions to reduce Chfr levels in order to allow cells to go through the G2/M checkpoint gate.

When Chfr cannot auto-ubiquitinate itself for destruction, its levels remain high. Likewise, pH3 levels are substantially reduced in the presence of such a protein. The FLAG-Chfr K2A protein for example, is resistant to ubiquitination-mediated degradation. It can ubiquitinate itself, but it cannot effectively reduce its own level. pH3 levels are substantially lower in cells expressing FLAG-Chfr K2A, in comparison to pH3 levels in cells expressing wild type FLAG-Chfr. The modification on the FLAG-Chfr K2A protein appears to improve the ability of this particular protein to keep chromosomes in a decondensed state. A possible explanation for this is that FLAG-Chfr-K2A binds to pH3 directly, auto-ubiquitinates itself or pH3 at this location, and the stabilized ubiquitin signal results in stearic hindrance that either prevents the chromosomes from compacting or prevents further access to pH3. In this way, pH3 levels could be kept low, and mitosis is prevented.

1.2. Roles of Chfr in the early prophase checkpoint and the spindle assembly checkpoints.

Several lines of evidence support the notion that CHFR is involved in two distinct mitotic checkpoints: the antephase or early prophase checkpoint, which guards entry into mitosis, and spindle assembly checkpoint, which guards the metaphase to anaphase transition. Both checkpoints are induced in response to microtubule damage.

The G2/M checkpoint and the spindle checkpoint should work together, with the G2/M checkpoint preventing damaged cells from progressing through the G2/M transition (lowering the EPI) and the spindle checkpoint catching anything that manages to squeeze through the G2/M checkpoint gate (increasing the MI). Unable to divide, these cells should then undergo apoptosis.

1.2.1 Chfr in the early prophase checkpoint. During microtubule damage induced by nocodazole, colcemid, or taxanes, CHFR has been shown to transiently delay the G2-to-M transition by mitotic index assay and immunocytochemistry (Scolnick and Halazonetis, 2000). The mitotic index, which is the percentage of cells with condensed chromosomes, is reduced in CHFR-expressing cells treated with microtubule poisons (Scolnick and Halazonetis, 2000; Summers et al., 2005; Ogi et al., 2005). Scolnick and Halazonetis (2000) analyzed DLD-1-neo and DLD-1-Chfr cells that were synchronized using the double thymidine block, and treated with nocodazole for 2 hours, 12 hours after release from the S phase block. The MI assay and immunocytochemistry was then done on both cell types to determine the effect

of Chfr expression. Colcemid-treated cells lacking Chfr (DLD-1-neo) progressed readily into mitosis like untreated cells, with no delay into mitosis upon nocodazole treatment. The mitotic index increased as cells progressed through mitosis, just like in untreated cells, and cells condensed their chromosomes faster than DLD-1-CHFR cells. These were also more prone to death during nocodazole exposure. Colcemid-treated cells over-expressing Chfr (DLD-1-Chfr), on the other hand, remained in an interphase-like state for several hours, with no chromosome condensation and low MI in comparison to untreated cells (Scolnick and Halazonetis, 2000). DLD-1 cells were also less prone to death during nocodazole-exposure. This indicates that Chfr may be involved in sensing microtubule damage at the G2/M transition, and signaling a response that allows cells to delay their entry into mitosis under conditions that could compromise genetic stability (Scolnick and Halazonetis, 2000). This transient delay was further characterized by Matsusaka and Pines (2004), who showed that cells not only could inhibit chromosome condensation but could actually decondense their chromosomes and enter back into an interphase-like state following treatment with colcemid during early prophase.

Kim et al. (2011) showed that when cells express an empty FLAG-vector, they are more likely to contain a strong anti-phospho-histone H3 signal after nocodazole treatment. When Chfr is expressed, however, the amount of phospho-histone H3 is less in comparison to the Chfr-lacking control. This indicates that Chfr prevents chromosome condensation during nocodazole exposure. These authors also showed that entry through the G2/M gate is associated with auto-ubiquitination, which could degrade the Chfr protein as cells enter mitosis.

1.2.1.1 Chfr, Cyclin B1-Cdc2, and Plk1. When microtubules are damaged, Chfr delays the cell cycle transiently by preventing Cyclin B1-Cdc2 from entering the nucleus, keeping it in the cytoplasm where it remains inactive (Summers et al., 2005). Cyclin B1-Cdc2 is the mitosis promoting factor (MPF). Kang et al. (2002) further showed that Chfr can inhibit the activation of Cyclin B1-Cdc2, and that Chfr can ubiquitinate Plk1 in *X. laevis* extracts. Their finding suggests that Chfr may delay entry into mitosis by ubiquitinating and reducing Plk1 levels, which in turn delays the activation of Cdc25c phosphatase and the inactivation of Wee-1 kinase, which in turn leads to a delay in Cdc2 activation (Cdc2 is part of the MFP complex), and delay in mitotic entry.

Furthermore, cells from Chfr knockout mice have been shown to over-express Plk1 in comparison to cells from wild type mice, which suggests that Plk1 is ubiquitinated by Chfr for degradation (Yu et al., 2005). This study, however, did not do any tests with nocodazole, which is supposed to activate the Chfr checkpoint. Other research, on the other hand, has not found a relationship between Chfr over-expression and reduced Plk1 protein levels (Matsusaka and Pines, 2004; Summers et al., 2005). Summers et al. (2005) show that Plk1 localizes to the centrosomes.

1.2.1.2 Chfr and Aurora A at early prophase. Another possible ubiquitination target for Chfr is Aurora A as this protein is capable of recruiting Cyclin B1 into the centrosomes (Hirota et al., 2003). Summers et al. (2005) found no relationship between Chfr over-expression and decreased Aurora A protein levels in HCT116 cells. However, they showed that when HCT116 cells expressing Chfr were treated

with nocodazole, cells that remained in interphase, and were presumably delayed by the CHFR checkpoint, did not contain active Aurora A at their centrosomes. This active form of Aurora A was only present in cells that had already overcome the checkpoint and were in late prophase. This data indicates that CHFR may function by inactivating Aurora A, a mitosis promoting protein, at the centrosomes at interphase. Chfr here may function through K-48-linked degradative poly-ubiquitination to inactivate Aurora A at interphase, and activate the same protein after the Chfr checkpoint is complete through K-63-linked poly-ubiquitination.

Yu et al. (2005) found that Aurora A is over-expressed in cells that have a CHFR knockout, indicating that Chfr may function to reduce Aurora A protein level and to keep this protein at a functional level. They also found that the C-terminus of Chfr interacts with Aurora A and that this interaction is required for Aurora A ubiquitination. These findings support the idea that Chfr functions through degradative K48-linked poly-ubiquitination. Privette et al. (2008b) also found that depletion of Chfr by siRNA results in Aurora A over-expression. This indicates that Chfr regulates Aurora A by degradation.

1.2.1.3 Chfr and p38 stress kinase. Another way in which Chfr may be able to induce delayed entry into mitosis in response to nocodazole treatment is through the p38 stress kinase pathway. The p38 stress kinase pathway has been shown to be activated by ubiquitination through K63-linked polyubiquitin chains (Wang et al., 2001).

Furthermore, treatment of cells with anisomycin or nocodazole activates the p38 stress kinases and results in a reduced mitotic index (Cano et al., 1994; Takeneka et al., 1998). Anisomicin is a p38 kinase activator. Matsusaka and Pines (2004) found that treatment of cells with anisomycin, colcemid, or nocodazole not only reduced the number of prophase cells but also caused cells in mid prophase to return to interphase. Inhibition of p38 with p38 kinase inhibitors abrogated this colcemid-induced checkpoint: cells entered mitosis in spite of the presence of microtubule stress, and they became arrested by the spindle checkpoint at the metaphase-to-anaphase transition. Injecting p38a and p38b stress kinases into early prophase cells resulted in the majority of cells returning to interphase, but injecting p38 δ and Erk2 had no such effect on the progression of these cells through prophase (Matsusaka and Pines, 2004). When the FHAΔ-Chfr dominant negative mutant was introduced into normal cells, the checkpoint was abrogated, but could be restored by injecting p38a into cells, causing them to return to interphase. This indicates that p38 probably acts downstream of Chfr in the Chfr checkpoint pathway.

The work done by Matsusaka and Pines (2004) also clearly points out that K-63-linked poly-ubiquitin chains may be involved over the more expected K-48-linked poly-ubiquitination during the G2/M checkpoint delay induced by microtubule stress. This group shows that ubiquitination is necessary for mitotic delay, but that degradative ubiquitination is not necessary for such a mitotic delay. They do this by inhibiting proteasomal degradation. In the presence of such inhibition, the chromosomes still delay their entry into mitosis. This means that

degradative ubiquitination is not necessary for the Chfr checkpoint. General ubiquitination, on the other hand, is necessary, for Chfr checkpoint delay.

1.2.2 Chfr in the spindle checkpoint. Aside from regulating the early prophase stage of mitosis, CHFR also appears to play a role in the later stage of mitosis, at the metaphase-to-anaphase transition, making sure that all chromosomes are properly attached to the mitotic spindle before the onset of anaphase. This transition is governed by the spindle checkpoint and regulates chromosome segregation and genetic instability (Yu et al., 2005).

CHFR deficient mouse cells, for example, spent a prolonged amount of time not only in prophase but also in anaphase. These cells were multinucleated, displayed increased aneuploidy, lagging chromosomes and failed cytokinesis (Yu et al., 2005).

Scolnick and Halazonetis (2000) distinguish clearly the early prophase checkpoint from the spindle checkpoint. Their data shows that DLD1-neo cells are stopped by the spindle checkpoint after exposure to nocodazole, whereas DLD1-Chfr cells are stopped initially by the early prophase checkpoint and then later by the spindle assembly checkpoint. This data shows a delay in cells expressing Chfr in comparison to those that do not express Chfr.

1.2.2.1 Chfr, BUBR1, and MAD2. In another study, cells treated with siRNA specific for Chfr also resulted in mitotic spindle checkpoint defects, including lagging anaphase chromosomes, multipolar mitotic spindles, tetraploid, binucleated giant

cells and misaligned chromosomes at the metaphase plate (Privette et al., 2008b). Furthermore, Chfr depletion by siRNA in these cells caused BUBR1 and MAD2, two mitotic spindle checkpoint proteins, to localize abnormally to the kinetochores during metaphase, causing impaired MAD2/CDC20 complex formation during microtubule stress. The MAD2/CDC20 complex prevents the APC from initiating anaphase prematurely, making sure that all of the sister chromatids are attached to the mitotic spindle apparatus (Privette et al., 2008b). Chfr, therefore, appears to function in the proper recruitment and activation of important spindle checkpoint proteins to the kinetochores. This is in line with K-63 linked poly-ubiquitination, which can act as a stress signal to alter protein localization or function (Newton et al, 2008).

So basically, taking the review of the literature into perspective, the G2/M checkpoint and the spindle assembly checkpoint should work together, with the G2/M checkpoint preventing damaged cells from progressing through the G2/M transition (lowering the EPI) and the spindle assembly checkpoint catching anything that manages to squeeze through the G2/M checkpoint gate (increasing the MI). Unable to divide, these cells should then undergo apoptosis or delay their division until conditions are favorable. A summary of the literature is presented in **Figure 1-2.**

1.2.2.2 Chfr and Aurora A. Chfr may also regulate proper spindle formation by regulating Aurora A protein levels. Aurora A is a mitosis promoting protein, localizing to the centrosomes, and is involved in centrosome maturation and spindle

formation (Privette et al., 2008b). If these structures do not mature and separate correctly, this could result in abnormal spindle formation and chromosome division. Privette et al. (2008b) showed that when Chfr levels are down, Aurora A levels are increased. Thus, abnormally low Chfr levels increase Aurora A levels, which increases centrosome number. Centrosomes are microtubule-organizing centers required for proper mitotic spindle formation. This means that abnormal Aurora A and Chfr protein levels could result in abnormal multipolar mitotic spindle structures, and aneuploidy.

Interestingly, one article showed that Aurora A could potentially recruit Ubc13 to the centrosomes during mitosis (Toland et al., 2003). This group showed that Ubc13 cannot localize to the centrosomes in the presence of mutated Aurora A, Ile31 variant of STK15. The article did not mention Chfr. Chfr, however, could in this way function by localizing to the centrosomes with Ubc13 and Aurora A to alter the behavior or stability of Aurora A by activation of that protein. The signal could be either K63-linked or K-48 linked in nature. The degradative signal could function first to reduce or inactivate Aurora A at the centrosomes, and the K63-linked signal could function to reactivate Aurora A at the centrosomes to allow mitotic progression to continue. Summers et al. (2005) showed that Aurora A is inactivated at the centrosomes in interphase in the presence of Chfr, and that the active form of Aurora A is only present in late prophase cells that have overcome the G2/M transition and progressed into mitosis. K-63 linked poly-ubiquitin chains could potentially be involved in such activation of Aurora A because a) Chfr can formulate

K63-linked polyubiquitin chains with Ubc13-Mms2; and b) Ubc13 can be recruited by Aurora A to the centrosomes (Toland et al., 2003).

1.2.2.3 Chfr and alpha-tubulin. Alpha-tubulin levels and acetylated alpha-tubulin levels may also be regulated by CHFR. Interestingly, when CHFR protein levels are reduced by CHFR siRNA knockdown, alpha-tubulin levels and especially acetylated alpha-tubulin levels are increased in expression. Furthermore, a ubiquitin signal appears to be associated with reduced alpha-tubulin levels and the presence of Chfr. This ubiquitin signal is reduced when CHFR is knocked down using the siRNA knockdown technique, and alpha-tubulin levels become noticeably higher during this knockdown. The signal appears to be a diubiquitin (Privette et al., 2008b). This supports the idea that Chfr can ubiquitinate other proteins, and that alpha-tubulin could possibly be yet another substrate for CHFR. Alpha-tubulin is an important component of the mitotic spindle. So Chfr in this case could reduce the level of this mitotic spindle component to guard proper spindle formation.

1.3 Reasoning behind thesis.

1.3.1 Introduction to Ubiquitination. Bothos et al. (2003) proposed that Chfr might delay entry into mitosis by ubiquitination, specifically by using the Ubc13-Mms2 ubiquitin-conjugating enzyme complex to catalyze the formation of K63-linked poly-ubiquitin chains.

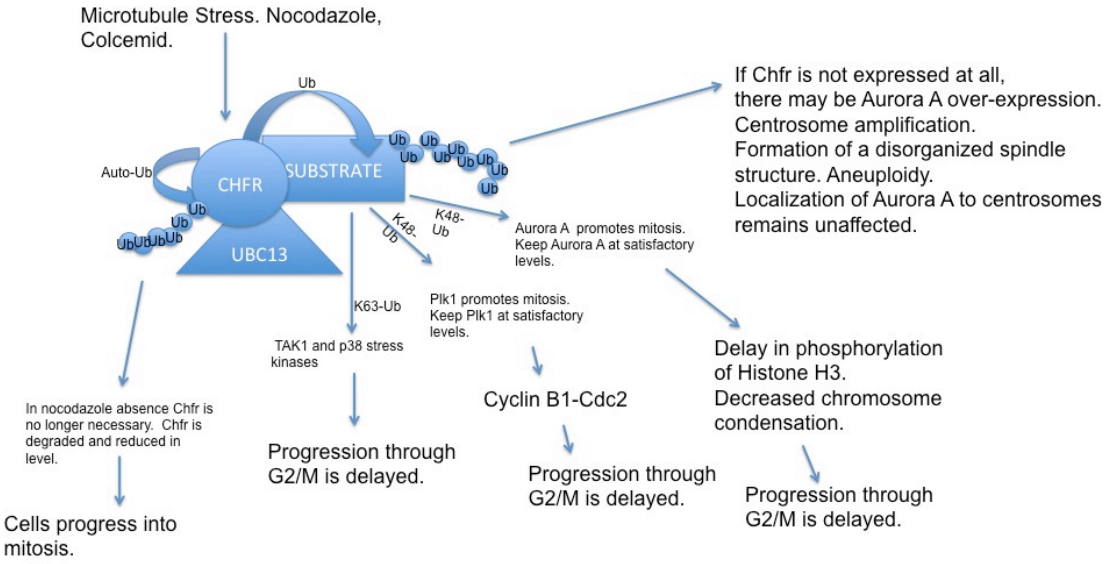


Figure 1-2. Some possible molecular pathways by which Chfr can attain the mitotic delay induced in response to microtubule poisons.

Ubiquitination is an enzymatic cascade that can alter the stability, behavior, or intracellular localization of a particular substrate protein (Newton et al., 2008). This control is achieved through the covalent attachment of a small molecule called ubiquitin (Ub) to the target's surface. The ubiquitination cascade consists of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-ligase enzyme (E3). Chfr, in this context, would be an example of an E3 enzyme, while Ubc13-Mms2 would be an example of an E2 enzyme complex. The E1 enzyme activates the Ub molecule, and passes it onto the E2 enzyme. The E2 enzyme can then attach the Ub molecule directly onto a target protein, with the E3 enzyme serving as an adaptor between the E2 and the target.

Alternatively, the E2 can also pass the Ub molecule onto the E3 enzyme, which can then pass the Ub molecule onto the target. Either way, the ultimate result is the ubiquitination of a particular substrate protein, which results either in protein degradation or in protein signaling. Target proteins can be mono-ubiquitinated, multiply mono-ubiquitinated on different lysine residues, or poly-ubiquitinated. The two most characterized types of poly-ubiquitin chains are the K63-linked and the K48-linked poly-ubiquitin chains. K48-linked chains mark faulty or unwanted proteins for destruction in the proteasome, whereas K63-linked chains mark a protein for cellular signaling and can alter protein activity.

Researchers are debating whether Chfr delays entry into mitosis through K-48 linked or K-63 linked poly-ubiquitin chains (Yu et al. 2005; Kang et a, 2002; Bothos et al., 2003). This ubiquitination could be either K-48 linked or K-63-linked in nature, with the majority of the literature leaning towards K-48 linked

degradative ubiquitination. Two reports support the K-63 linked poly-ubiquitination of Chfr. Bothos et al. (2003) proposed that the Chfr protein and the Ubc13-Mms2 protein complex work together to mark a particular substrate for cellular signaling via K-63 linked poly-ubiquitin chains, resulting in delayed mitotic entry following microtubule damage. Matsusaka and Pines (2004) further showed that degradative ubiquitination is not necessary for the Chfr checkpoint delay, indicating K-63 linked poly-ubiquitin chains may be involved in this process instead. Kim et al. (2011) provided an alternative hypothesis, that K-48 linked poly-ubiquitin chains are involved instead. The bottom line is that Chfr accumulates in the nucleus and interacts with DNA to somehow influence chromosome dynamics. The signal that is generated must somehow result in decondensed chromatin.

1.3.2 Hypothesis and rationale behind the hypothesis. Based on the literature, the hypothesis of this project is that Ubc13, Mms2/Uev1 and Chfr function in mitosis at the G2/M transition, and that these proteins regulate entry into mitosis through ubiquitination.

Chfr predominantly appears to be a nuclear protein, localizing to interphase nuclei. This nuclear localization is in line with the reported Chfr function, which is that of chromosome decondensation, because an interphase nucleus has decondensed chromatin. Chfr could potentially bind to DNA directly and interact with proteins that define the transition from interphase (G2) to early prophase (M), and are important for proper mitotic entry. At this point, Chfr could bind to condensing chromatin and use Ubc13-Mms2/Uev1 to conjugate K48-linked or K-63-

linked poly-ubiquitin chains. The ubiquitin signal on Chfr could physically interfere with chromosome compaction. Though potentially degradative in nature, the signal could also contain an immediate bulky property that temporarily interferes with DNA condensation, increasing the likelihood of a more decondensed form of DNA at the interphase-early prophase stage, resulting in temporary chromosome decondensation at interphase. When not necessary, Chfr could auto-ubiquitinate itself for destruction at the interphase/early prophase stage to allow cells to enter mitosis, or mark important cell cycle promoting proteins for destruction. Either at the kinetochores, where microtubules attach to DNA, or at the centrosomes where microtubules originate from. Following mitotic delay, Chfr, Ubc13, and Mms2/Uev1 could then generate K63-linked poly-ubiquitin chains to activate important cell cycle promoting proteins, either at the DNA site or at the centrosomes.

The main objectives of this project were to develop a hypothetical model by which Chfr, Ubc13, and Mms2/Uev1 could function during mitosis, analyzing protein interactions, protein localization, protein level, protein modification, and detection of protein function.

CHAPTER 2: MATERIALS & METHODS

2.1 Immunocytochemistry.

SAOS2 cells were fixed and permeabilized with -20°C methanol for 5 minutes, and were then incubated with the mouse 4E11 Ubc13-specific antibody and the rabbit Chfr-CR antibody (sc-28263; Santa Cruz Biotechnology) overnight. The DAPI stain was not applied. The photos show an early prophase cell, and a metaphase cell, and a telophase cell, all of which have chromosomes with 4E11 immunoreactivity stronger than their surroundings. Alternatively, SAOS2 cells were washed with PBS, fixed with 4% formaldehyde for 30 minutes, and permeabilized with methanol for 5 minutes, after which they were stained with the Chfr-CR antibody and the mouse Ubc13 antibody.

2.2 siRNA knockdown.

iRNA specific for Ubc13 (5575; GenePharma) was dissolved in RNAse-free water. iUbc13 with an optical density of 1 OD was added to 150 µl of water to get a stock solution od 20 nM. Control iRNA had an optical density of 0.5 OD and was added to 7.5 µl of water to get a stock concentration of 20 nM. The solutions were frozen in 10 µl alliquotes and refrozen a few times only. The RNAiMAX reagent protocol was followed by knockdown of Ub13. Cells were seeded into a 35 mm tissue culture dish, with 1.5 ml of media at a 50 % confluency. Cells should be plated at a confluency of 50% if they grow quickly and a higher confluency if they grow slowely. 5 ml of media was removed the next day leaving 1.0 ml of media. Fresh

media should not be added. 1.5 µl of Ubc13 siRNA was mixed with 250 µl of OPTIMEM and the solution was allowed to sit for 5 minutes at room temperature. 4 µl of RNAiMAX Reagent was mixed with 250 µl of OPTIMEM, and the solution was allowed to sit for 5 minutes at room temperature. siRNA and RNAiMAX solutions were allowed to sit for 20 minutes. 500 µl of the Ubc13 siRNA-iMAX Reagent solution was then added to the 1 ml culture, leaving a final volume of 1.5 ml. Cells were then incubated overnight. Alternatively, media can be removed after 6 hours. Maximum activity of the Ubc13 siRNA appears to be at about 3 days after transfection.

2.3 <u>Coimmunoprecipitation and Western blot analysis.</u>

Preparing the cell lysate: Four 10 cm dishes of SAOS2 cells, grown in 10ml of SIGMA DMEM + 10% FBS, were rinsed with PBS and lysed with 300 µl of lysis solution. Cell lysates were sonicated, clarified by centrifugation on maximum speed for 20 minutes to remove cell debris, and pre-absorbed with sepharose beads to reduce non-specific binding. For pre-absorption, the lysate was diluted 1:5 in IP diluent, and the PBST-washed beads were added to the diluted cell lysate. The diluted lysate and bead mixture was then incubated for 4-6 hours. The beads used for clarification were then removed by centrifugation at 2000 x *g* for 2 minutes.

Making the bead-antibody complex: A fresh batch of beads was then washed several times with PBST and the beads were blocked for 1hr with a 50mg BSA powder/1ml of PBST solution while rotating at 4°C. 2-5 µl of mouse 4E11 antibody and 10 µl of 1% thimerosal was then put into the appropriate bead-

containing tubes. The blocked beads were then incubated with the antibody for approximately 4-6hrs to form a complex. The bead-antibody complex was then further washed with more blocking solution to remove excess antibody.

Application of cell lysate to bead-antibody complex: Clarified lysate was then applied to the bead-antibody complex; and the tubes were placed horizontally on a gently rocking platform on ice overnight at 4°C. Samples were then spinned down at 2000xg for 2 minutes and washed with PBST at least 8 times.

Preparation of sample for Western blot analysis: Elution buffer was then added to the bead-antibody complex, samples were boiled for 5 minutes, and 6µl of iodoacetamide was added to each tube after boiling. Samples were then spinned down on maximum speed to compact the beads, and the supernatant was transferred to fresh tubes. 10µl of sample was then loaded in each well of a 1.0mm, 12% gel. The samples on the gel were then transferred onto a membrane. The membrane was blocked with a 5% skim milk/PBST/0.01% thimerosal solution and probed with the mouse 4E11 antibody (1:5000) for at least 4 hours. The membrane was then washed 4 times for 10 minutes with PBST. A goat anti-mouse IgG HRP conjugate (1:10,000) was used as the secondary antibody.

Lysis Solution: 200 µl of phosphate buffer (0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 ml ddH_2O); 54.8 µl 5M NaCl; 20 µl of phosphate inhibitory cocktail; 20 µl 1M NEM in DMSO; 40 µl 10% SDS; 40 µl 10% deoxycholate; 1625ml water.

IP diluent: 1000 µl of phosphate buffer (0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 ml ddH_2O); 274 µl 5 M NaCl; 100 µl phosphate inhibitory cocktail; 100 µl 1M NEM dissolved in DMSO; 8526 ml of water.

Elution buffer: 125 µl loading buffer; 25 µl phosphate buffer (0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 ml ddH_2O); 50 µl 10% SDS (1% final); 5 µl 1M DTT; 295 µl autoclaved ddH_2O; 6 µl 0.5 M iodoacetamide per tube.

2.4 <u>Mitotic shake off procedure.</u>

A mitotic shake-off experiment was carried out in nocodazole-treated and untreated HeLa-Chfr cells. Cells were either treated with or left untreated with nocodazole (for O/N), and the rounded cells were shaken off, separating the flat cells from the rounded cells. The rounded cells are cells in a late prophase-like stage of mitosis (fraction 2+4; corresponding to lanes 2 and 4), whereas the flat cells represent cells that are either in interphase or in early prophase (fractions 1+3; corresponding to lanes 1 and 3), with some possible late-prophase-like cell contamination. Cells were lysed, and lysates analyzed by Western blot analysis. Ubc13, Mms2, and Chfr protein levels were compared in each of the four cells fractions: treated cell fraction 1 and 2, untreated cell fraction 3 and 4. The Western blots were probed for Chfr using the Myc-tag antibody. For Ubc13 using the 4E11 antibody, and for Mms2/Uev1 using the 2H11 antibody.

Four 10 cm dishes of 95% confluent HeLa-Chfr cells grown in 10 ml of SIGMA DMEM + 10% FBS were used for the mitotic shake off experiment. Nocodazole was added at a concentration of 0.5 µg/ml to one of the 10 cm dishes. The nocodazole-treated cells were then divided into interphase-early prophase (INT/EP) cell fractions and late prophase (LP) cell fractions, which were rounded in shape. The three untreated plates were also divided into the same two cell fractions:

interphase-early prophase and late prophase. Rounded cells representing cells in late prophase were dislodged from the monolayer by using a pipette. The four cell fractions were then washed with PBS and lysed with 300 µl of lysis solution. The samples were then analyzed by Western blot analysis using a 1.0 mm, 12% acrylamide gel. The membrane was probed using a mouse antibody that recognizes both Mms2 and Uev1a (2H11, 4:5000), a mouse antibody specific for Ubc13 (3:10,000), and a rabbit myc-tag antibody (06-549, UPSTATE, 3:10,000) specific for myc-tagged Chfr.

Lysis solution: 125 µl of phosphate buffer (0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 ml ddH_2O); 50 µl of 1M NEM; 50 µl of protease inhibitory cocktail; 62.5 µl of 10% SDS; 125 µl 10% deoxycholate; 4.525 ml PBS.

2.5 <u>Cell Culture and Synchronization.</u>

Cells were cultured in SIGMA DMEM + 10% FBS. U2OS cells were synchronized using the double thymidine, single R03306 block. Cells were treated with 2mM thymidine for 21 hours, released for 12 hours, treated with 2mM thymidine for 21 hours. The thymidine was then removed and cells were immediately treated with 10 µM of R03306 for 22 hours. R03306 is a Cdk1 inhibitor and blocks cells at the G2/M gate. The R03306 drug was then removed and the cells were released from the G2/M block for 0 min, 10 min, and 45 minutes to watch progression through the early prophase stage of mitosis. Most U2OS cells enter early prophase 10 minutes after release from the G2/M block. Photos were taken under the red and blue fluorescent filter and merged with phase contrast photos.

Lipofectamine RNAiMAX Reagent (13778-030; Invitrogen) and OPTI-MEM®1 (31985-062; GIBCO Invitrogen) were used for the transfections.

CHAPTER 3: RESULTS

3.1 Chfr expression in HeLa cells.

The first objective was to determine whether HeLa-Chfr cells, which should contain a myc-tagged Chfr vector, do indeed over-express the Chfr protein. Chfr expression in these cells was confirmed using Western blot analysis (WBA) and immunocytochemistry (ICC) **(Figure 3-1).** HeLa-Chfr cells express myc-tagged Chfr, whereas HeLa control cells do not express this protein. The actin control indicates equal loading. Another possible control that could be useful for this experiment could be a HeLa cell line expressing only a Myc-tag containing vector. The Myc-tag antibody localizes predominantly in the nucleus. Cells in interphase had Myc-Chfr in the nucleus.

3.2 Protein-Protein interaction between Chfr and Ubc13.

A physical relationship between Chfr, a mitotic checkpoint protein, and Ubc13 *in vivo* through coimmunoprecipitation is shown in **Figure 3-2.** A Ubc13-specific antibody immobilized on sepharose beads was used to precipitate Ubc13 out of HeLa-Chfr cell extracts. The Myc-tag antibody was used to demonstrate that Chfr coimmunoprecipitates with Ubc13. **Figure 3-2** clearly shows that Chfr

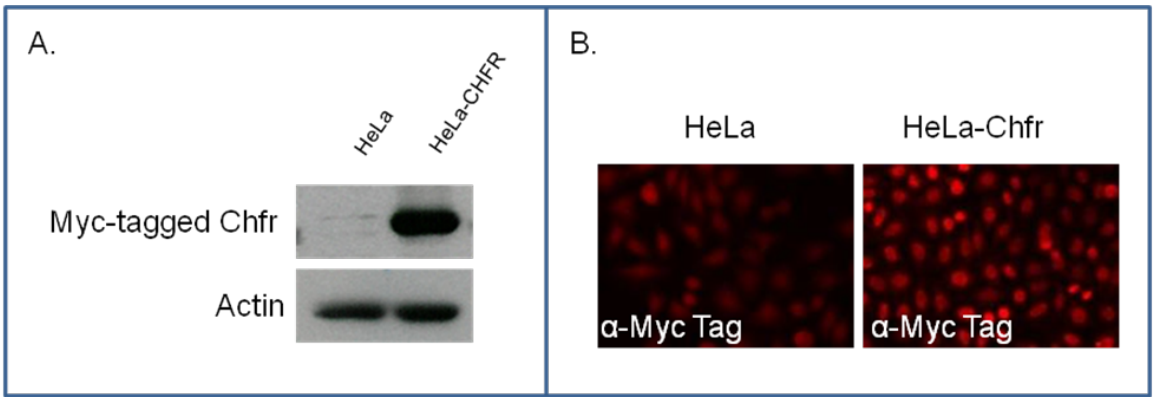


Figure 3-1. Western blot (A) and immunocytochemistry (B) showing over-expression of myc-tagged Chfr in HeLa cells. HeLa and HeLa-Chfr cells were grown in 10 cm dishes, and lysed using lysis solution. Lysates were prepared and boiled for 5 minutes for Western blot analysis. The rabbit anti-Myc-tag antibody and a secondary rabbit antibody was used to detect Myc-tagged Chfr for both experiments. Chfr localizes to the interphase nucleus and also to telophase nuclei.

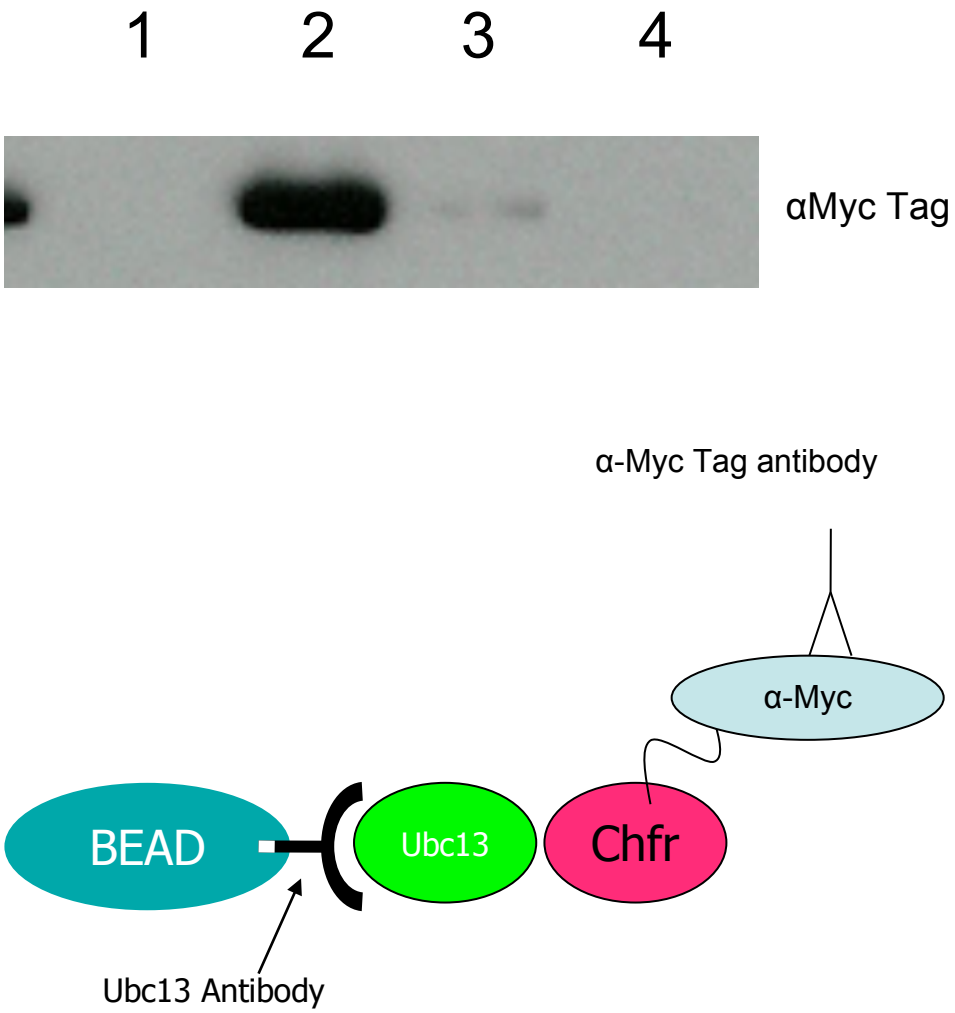


Figure 3-2. Myc-tagged Chfr coimmunoprecipitates with Ubc13 in HeLa-Chfr cells. Ubc13 was pulled out of HeLa cells over-expressing Myc-tagged Chfr using a Ubc13-specific antibody conjugated to protein G-sepharose beads, and the blot was probed for Myc-tagged CHFR, followed by a secondary HRP-conjugated rabbit antibody. (**Lane 1**) Beads; no lysate; Ubc13 antibody. (**Lane 2**) HeLa-Chfr whole cell lysate. (**Lane 3**) Beads; HeLa-Chfr whole cell lysate; Ubc13 antibody (**Lane 4**) Beads; HeLa-Chfr whole cell lysate; no Ubc13 antibody. The coimmunoprecipitation indicates that the Chfr and Ubc13 proteins interact together in cells expressing Myc-tagged Chfr.

coimmunoprecipitates with Ubc13 indicating these two proteins interact together. The interaction is faint.

3.3 A potential method for Chfr checkpoint detection.

3.3.1 Early prophase index assay. Another part of the project was focused on the early prophase index (EPI) assay tailored for Chfr checkpoint analysis. The Chfr checkpoint is measured either by live microscopy in individual cells or by using the mitotic index assay (Matsusaka and Pines, 2004; Bothos et al., 2003). Mitotic index assay is an indirect way of detecting Chfr checkpoint activity. In order to unravel the molecular mechanism by which any protein functions, one must first detect an effective way of detecting its activity. This was done by developing an effective way of detecting cells at the early prophase (EP) stage of mitosis -- because that is the point at which Chfr has been thought to specifically exert its effects. The general idea behind the assay is that a high number of EP cells following microtubule damage means failure to delay mitosis and indicates the absence of Chfr checkpoint activity. A *low* number of EP cells following microtubule damage, on the other hand, indicates effective mitotic delay, and Chfr activity *presence.* Currently, researchers are detecting Chfr checkpoint activity by counting the *entire* mitotic index of a cell population. This is an indirect way of assessing Chfr checkpoint activity because it focuses on the entire mitotic cell population (arrested by the spindle checkpoint) instead of looking *exclusively* at the EP stage of mitosis where Chfr specifically exerts its effects. In other words, the mitotic index is the percentage of cells that squeeze

through the checkpoint. The early prophase index (EPI) assay is thus a more direct way of assessing Chfr checkpoint activity and could provide resolution between the early prophase checkpoint and the spindle assembly checkpoint.

3.3.2 Visually defining the early prophase stage of mitosis. Using DAPI staining is ineffective at detecting early prophase (EP) cells as cells at this stage have not yet fully condensed their chromosomes and may appear as though they are still in interphase **(Figure 3-3).** To efficiently detect EP cells, and to distinguish them from interphase and late prophase (LP) cells, I used the α-phospho-histone H3 (α-pH3) antibody and the α-nucleolin antibody **(Figure 3-4).**

The α-pH3 antibody is a useful marker for identifying mitotic cells because it detects phosphorylated histone H3. The phosphorylation of this histone is necessary for chromosome condensation and occurs rapidly during the very early stages of mitosis, even before the chromatin is fully condensed. This allows us to detect EP cells and to distinguish these cells from interphase cells. The α-nucleolin antibody, on the other hand, stains the nucleoli of cells. Nucleoli disappear as cells enter into the late prophase (LP) stage of mitosis. Being able to detect the presence or absence of nucleoli, therefore, allows one to distinguish early prophase cells from late prophase cells. Used in combination, these two markers helped to clearly define the EP stage of mitosis and the point at which Chfr functions **(Figure 3-5).**

3.3.3 Sensitization by synchronization. Though the EPI assay appears to be applicable to SAOS2 and U2OS cells, it is not very sensitive, and will not be better than the mitotic index (MI) assay unless we can effectively increase the number of early prophase cells for analysis in each cell population.

To improve the assay, I needed a synchronization method that would allow me sensitive control over the G2/M entry gate, so that upon release, the cells would enter mitosis rapidly together.

This task was a challenge because currently there are no effective synchronization methods out there for studying mitotic entry in mammalian cells. There are methods in the literature that block cells at the G1/S gate, such as the thymidine-aphidicolin or thymidine-cytidine block (Scolnick and Halazonetis, 2000; Summers et al., 2005). There are also methods in the literature that block cells in the middle of mitosis, by using nocodazole or a combination of the R03306 drug with nocodazole (Vassilev et al., 2006). For the study of early mitotic entry, however, there does not appear to be any effective methods of synchronization. The R03306 drug can be used alone to synchronize cells at the G2/M gate (Vassilev et al., 2006) but this method does not always synchronize cells as effectively as I would like (approximately 15-30%).

For this reason, a method for synchronizing mammalian cells was developed, referred to here as the "double thymidine, single R03306 block" (2xThymidine-1xR03306). This technique is essentially a double G1/S, single G2/M block. To synchronize cells, cells are treated with 2mM thymidine for 21 hours, released for

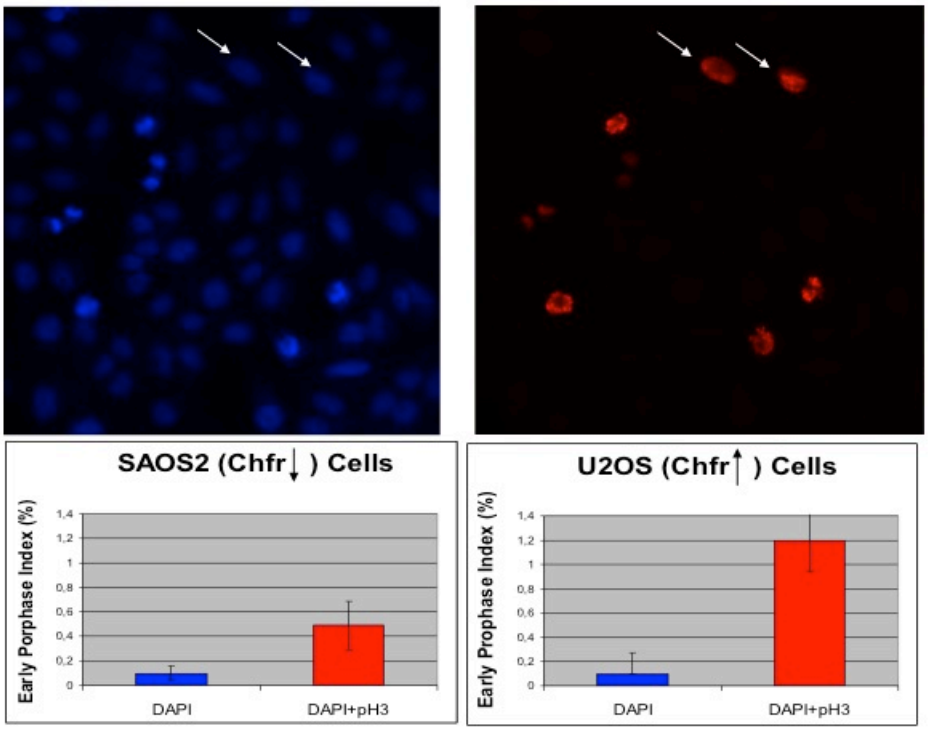


Figure 3-3. DAPI used in combination with the anti-phospho-histone H3 (α-pH3) rabbit antibody is more effective at detecting early prophase cells than the DAPI stain alone. The α-pH3 antibody detects early prophase cells more accurately than the DAPI stain (A, B), which results in a higher and more accurate early prophase index (EPI) in untreated SAOS2 and U2OS cells (C, D). SAOS2 cells are expected to express the Chfr protein, and U2OS cells are not expected to express the Chfr protein, in comparison to SAOS2 cells. Arrows point to early prophase cells, which can be easily overlooked by staining only with DAPI.

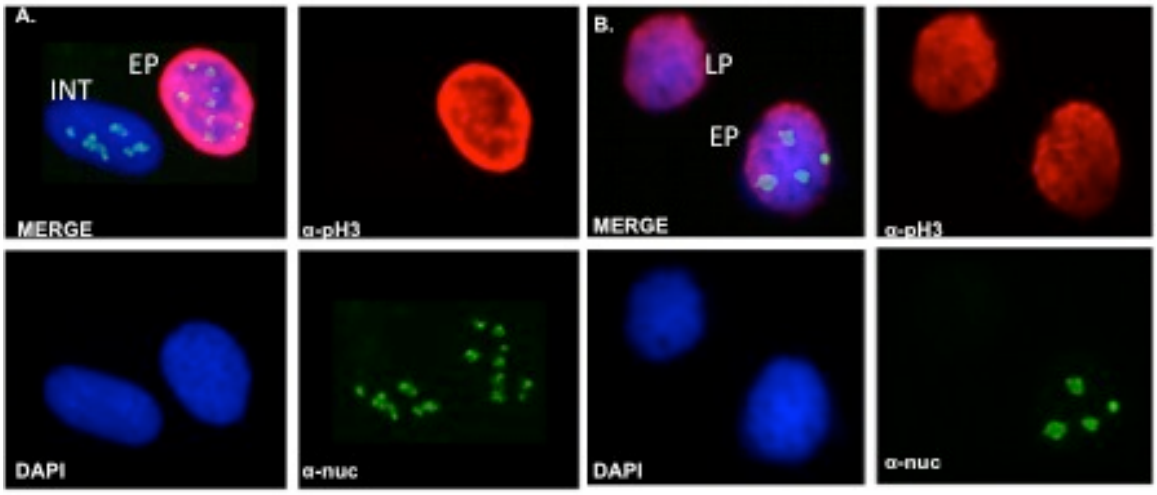


Figure 3-4. Visually defining the early prophase stage of mitosis. (A) The α-phospho-histone (α-pH3) rabbit antibody can distinguish early prophase cells from interphase cells. (B) The α-nucleolin (α-nuc) mouse antibody can distinguish early prophase cells from late prophase cells. EP = early prophase; LP = late prophase; INT = interphase.

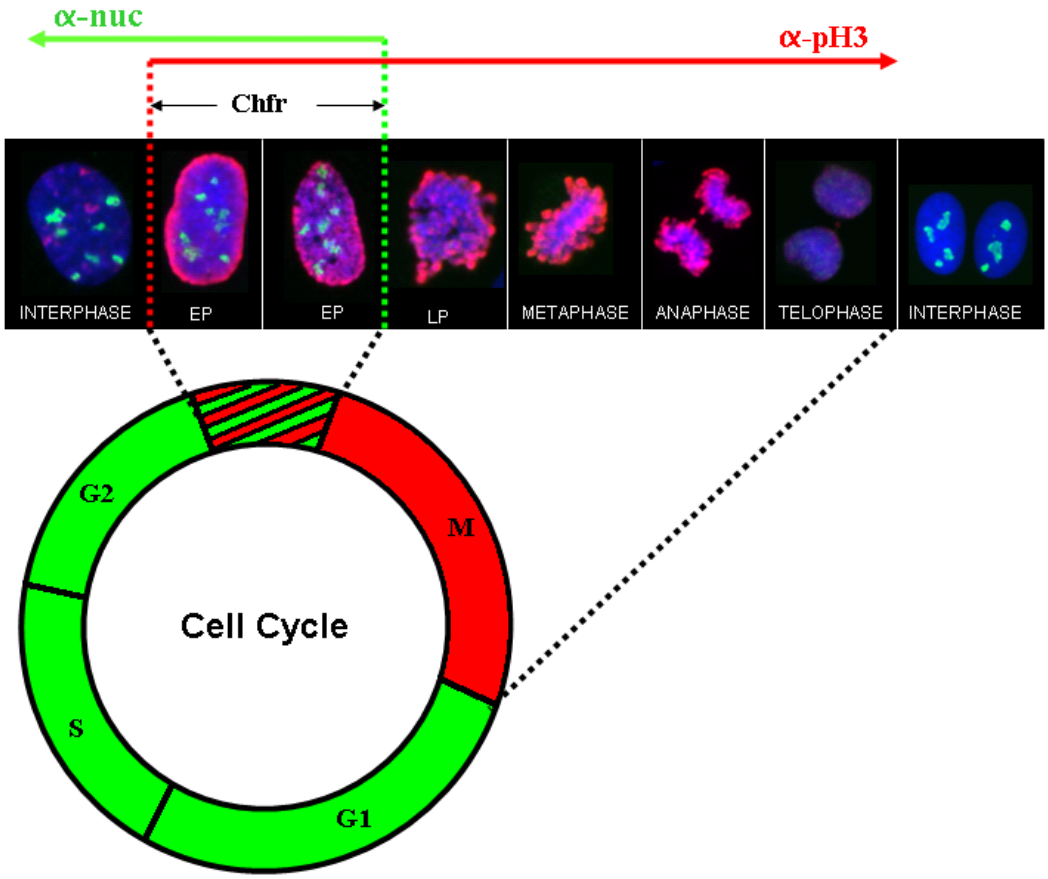


Figure 3-5. Visually defining the early prophase stage of mitosis. The DAPI (blue), α-phospho-histone H3 (red), and α-nucleolin (green) stains visually define the period during which the Chfr checkpoint functions. EP = early prophase; LP = late prophase α-nuc = anti-nucleolin; α-pH3 = anti-phospho-histone H3.

12 hours, and treated with 2mM thymidine for 21 hours. This should synchronize them at the G1/S phase of the cell cycle. The thymidine is then removed and cells.

For this reason, a method for synchronizing mammalian cells was developed, referred to here as the "double thymidine, single R03306 block" (2xThymidine-1xR03306). This technique is essentially a double G1/S, single G2/M block. To synchronize cells, cells are treated with 2mM thymidine for 21 hours, released for 12 hours, and treated with 2mM thymidine for 21 hours. This should synchronize them at the G1/S phase of the cell cycle. The thymidine is then removed and cells are immediately treated with 10 μM of R03306 for 22 hours. R03306 is a Cdk1 inhibitor and blocks the cells at the G2/M gate (Vassilev et al., 2006). This way, cells that have accumulated at the G1/S boundary can now progress and accumulate at the G2/M boundary. R03306 is then removed, cells are released, and should now progress synchronously through the early stages of mitosis**.**

HeLa, HeLa-Chfr, SAOS2, and U2OS cells were then synchronized by using this method. U2OS cells synchronized the best. In one experiment, 50.8% of U2OS cells entered mitosis within 45 minutes of release from the G2/M block; and in another experiment, 64.6% of cells entered mitosis upon release **(Figure 3-6).** The mitotic index was scored as the percentage of rounded cells in a cell population using phase contrast microscopy. As a control, U2OS cells were also treated with R03306 alone. Upon release from the G2/M block, only 27.4% of cells entered mitosis in one experiment; and in another experiment, 28.3% percent of cells entered mitosis upon release. To look at early prophase cells specifically, the experiment was then repeated and the cells released for 0 minutes, 10 minutes, and

45 minutes from the 2xThymidine-1xR03306 block and stained the cells with the anti-phospho-histone H3 antibody, which detects mitotic cells **(Figure 3-7)**. At the 0 minute time point, very little cells are in mitosis. The phase contrast photo in the 0 minute panel shows clearly that the nucleoli of these cells have not yet broken down, which indicates that they are blocked at a stage where the Chfr checkpoint should still be active according to Matsusaka and Pines (2004).

After 10 minutes of release from the G2/M block, many U2OS cells were visible at the early prophase stage of mitosis. At the 45-minute time point, most cells were in late prophase. The next step will be to try and optimize the condition for this U2OS cell line as I noticed some dead cells and fragmented nuclei in the 0 min control **(Figure 3-8).**

Synchronized HeLa-Chfr cells had 44.7% and 54.4% of cells entering mitosis upon release from the 2xThymidine-1xR03306 block in two separate experiments. Following release from treatment with R03306 alone, resulted in 14.7% and 35.7% of cells entering mitosis. Synchronization of HeLa cells with the 2xThymidine-1xR03306 block was also efficient, with 33.9%, 35.1%, and 30.7% of cells entering mitosis in three separate experiments upon release. These numbers, however, did not vary greatly when compared to the percentage of HeLa cells treated with R03306 alone (27.4%, 26.2%).

SAOS2 cells were the least effective at synchronization by the 2xThymidine-1xRO3306 block, but the method still managed to increase the number of cells

entering mitosis to 28.7%. The single R03306 control treatment for SAOS2 cells has not yet been done.

The 2xThymidine-1xR03306 synchronization method could prove to be a superior method of synchronizing cells for the study of mitotic events.

Firstly, the method gives a sensitive control over the G2/M entry gate, allowing for rapid entry into mitosis within 10-45 minutes of release from the G2/M block. Secondly, the method allows rapid entry of a significantly larger number of cells to enter mitosis. Thirdly, the method appears to be better than a single R03306 block alone, as far as U2OS cells are concerned. Fourthly, the 2xThymidine-1xR03306 block is better than a mere 2xThymidine-G1/S block, which synchronizes cells at an undesirable stage of the cell cycle for the study of early mitotic events. Fifthly, the 2xThymidine-1xR03306 block does not require nocodazole for synchronization. Many researchers use the 2xThymidine block in combination with nocodazole, to get a large (90%) population of cells in "mitosis." This combination method, however, will synchronize cells in the middle of mitosis, not at the beginning, and for this reason is not useful for studying early mitotic events, such as the early prophase Chfr checkpoint. Secondly, nocodazole is a microtubule poison, so it is undesirable for synchronization if you are trying to study events that are induced by microtubule stress in the first place, such as the Chfr checkpoint. Thirdly, cells "synchronized" with nocodazole, do not necessarily progress "synchronously" or normally through the rest of mitosis (Cooper et al., 2006) For these reasons, the 2xThymidine-1xR03306 block may be a useful synchronization technique for studying early mitotic events.

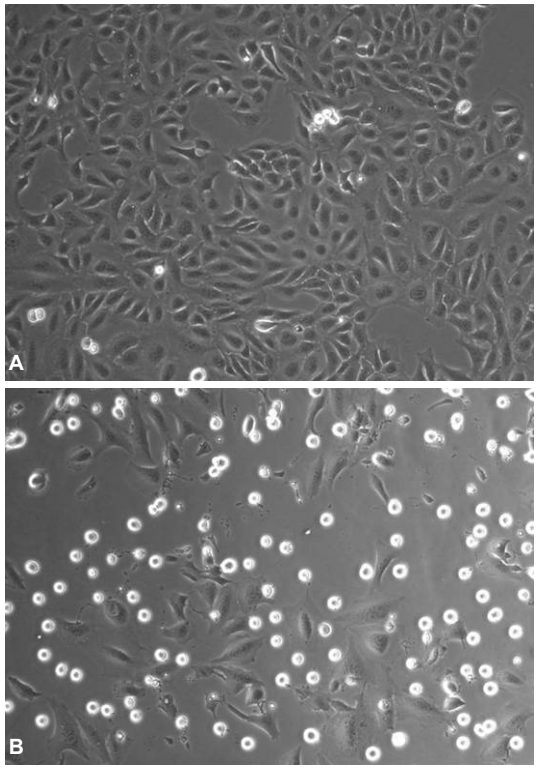


Figure 3-6. U2OS cells synchronized by the double thymidine, single R03306 block. In one experiment, 50.8% of U2OS cells entered mitosis within 45 minutes of release from the G2/M block; and in another experiment, 64.6% of cells entered mitosis upon release. Mitotic index was scored using phase contrast.

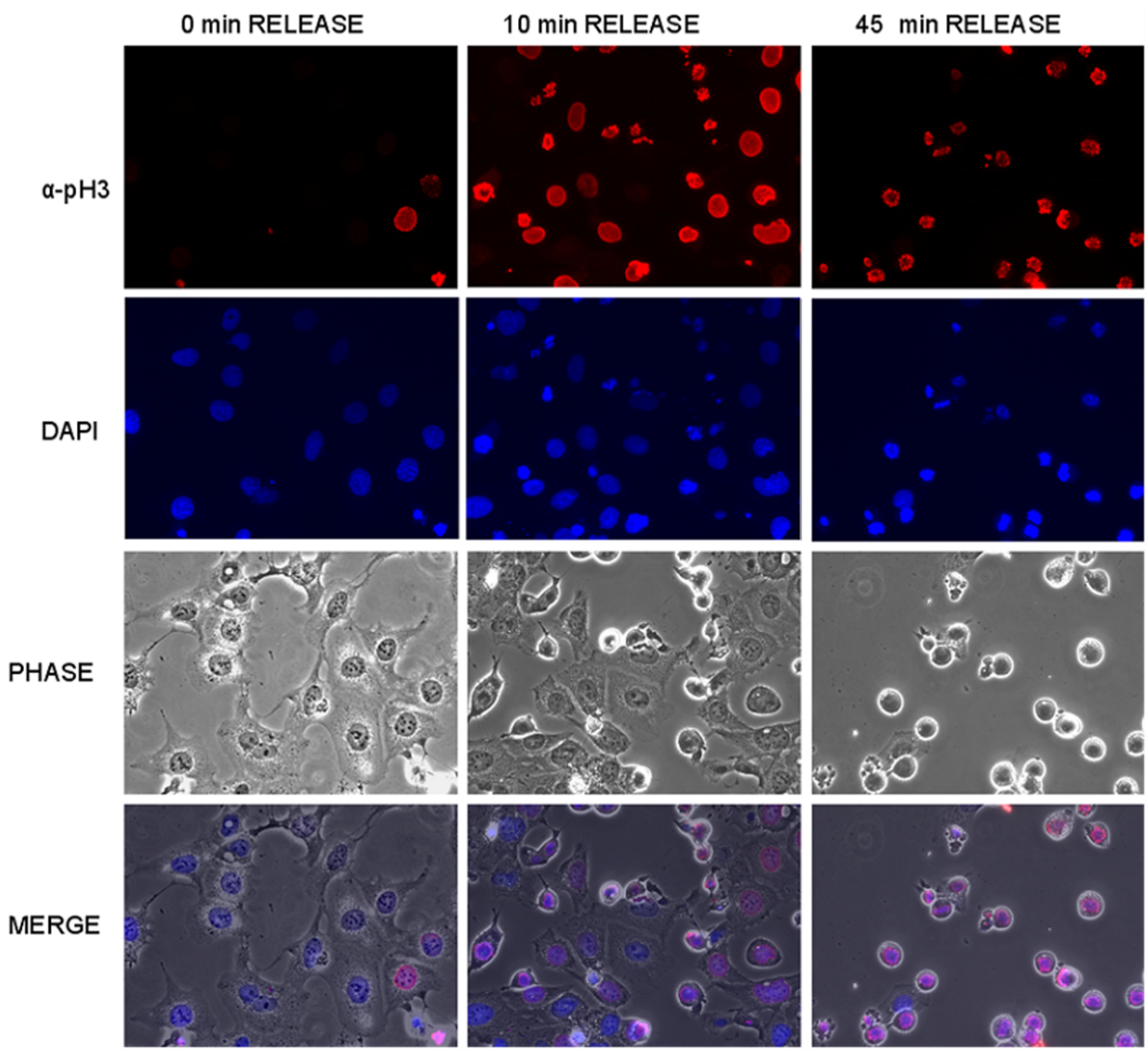


Figure 3-7. Synchronization of U2OS cells at the early prophase stage using the double thymidine, single R03306 block. U2OS cells were synchronized using the double thymidine, single R03306 block. Cells were treated with 2mM thymidine for 21 hours, released for 12 hours, treated with 2mM thymidine for 21 hours. The thymidine was then removed and cells were immediately treated with 10 μM of R03306 for 22 hours. R03306 is a Cdk1 inhibitor and blocks cells at the G2/M gate. The R03306 drug was then removed and the cells were released from the G2/M block for 0 min, 10 min, and 45 minutes to watch progression through the early prophase stage of mitosis. Most U2OS cells enter early prophase 10 minutes after release from the G2/M block. Photos were taken under the red and blue fluorescent filter and merged with phase contrast photos.

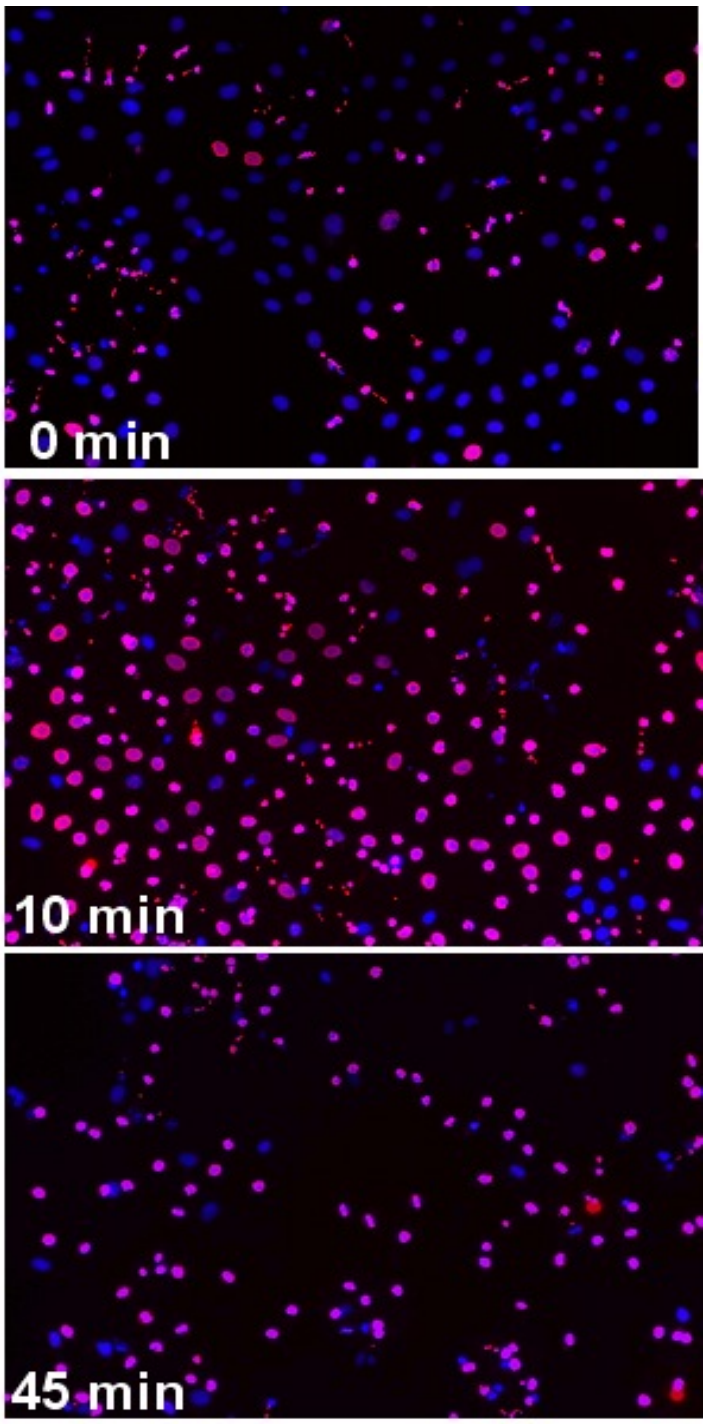


Figure 3-8. Synchronization of U2OS cells at the early prophase stage using the double thymidine, single R03306 block. Cells were synchronized as above. Photos were taken under the red (anti-phospho-histone H3 antibody) and blue (DAPI) fluorescent filters, and the images were then merged together.

One worry for this particular study is that blocking cells at the G2/M transition using the RO3306 Cdk1 inhibitor (Vassilev et al., 2006) stops cells too late in the cell cycle. So by the time that the cells reach this particular point in their cycle, Chfr may no longer be able to act on them, as they could have already passed through the Chfr checkpoint gate. The phase contrast photo in **Figure 3-7**, however, indicates that, this method should synchronize cells effectively for G2/M checkpoint analysis because the cells blocked at the 0 minute time point contain intact nucleoli. The checkpoint functions when colcemid is added before the nucleoli break down, but is no longer functional after the nucleoli break down (Matsusaka and Pines, 2004). If this method of synchronization cannot be applied to the study of Chfr checkpoint activity at the EP stage of mitosis, it could still be a useful and superior method of synchronization for other early mitotic studies, such as nuclear envelope breakdown, nucleolar breakdown, phosphorylation of histone H3, chromosome condensation, centrosome separation, and spindle formation. This synchronization could also be used to collect cells at later stages of mitosis without the damaging effects of microtubule poisons.

3.4 Detecting Chfr checkpoint activity in various cell lines.

3.4.1 HeLa and HeLa-Chfr cells. The mitotic index assay was used to determine whether HeLa-Chfr cells contain Chfr checkpoint activity. One experiment was done using unsynchronized cells and another was done after synchronization. **Figure 3-9a** shows that the MI of unsynchronized HeLa-Chfr cells was very similar to that of

HeLa control cells following nocodazole treatment. The effect of nocodazole was very similar on both cell types. Synchronized HeLa and HeLa-Chfr cells showed the same profile, with both cell types entering mitosis rapidly upon release from the G2/M block, in the absence of nocodazole **(Figure 3-9b).** The effect of nocodazole presence reduced the mitotic index slightly.

3.4.2 U2OS, U2OS-GFP, U2OS-EGFP, and U2OS-EGFP-Chfr. U2OS cells stably expressing EGFP-Chfr were also generated. This has previously been done (Scolnick and Halazonetis, 2000; Bothos et al., 2003) where HA-tagged Chfr was transfected into U2OS cells, resulting in a significant decrease of mitotic index after nocodazole treatment. Fluorescent clones were selected using geneticin and a Western blot was run to determine which selected clones are expressing a protein of the correct size **(Figure 3-10a).** From the 11 putative clones, Clone 7 and 8 contained bands with an expected size between 75 and 100 kDa, so these two clones were selected to do the MI assay. U2OS, U2OS-GFP, and U2OS-EGFP-Chfr cells were then treated with nocodazole **(Figure 3-10b).** U2OS-EGFP-Chfr clone 8 has a smaller MI in comparison to the untransfected. U2OS control cell line **(Figure 3-10b).** U2OS-EGFP-Chfr Clone 7 also had a smaller MI in comparison to the untransfected U2OS control cell line. This reduction indicates possible Chfr checkpoint activity. However, the mitotic indexes of Clone 7 and 8 were higher than the U2OS-GFP control, indicating no effect of Chfr presence on mitotic index.

Even though the U2OS-GFP control did not work for this experiment, it should be noted that Clone 7 had a much higher EGFP-Chfr expression than Clone 8. These results are in line with the Western blot **(Figure 3-10a).** When compared to clone 8, the mitotic index of Clone 7 is reduced here, and is much smaller in comparison to the MI of Clone 8. The more Chfr expression there is, the more reduced the MI should should reduce MI and entry into mitosis. This indicates that the EGFP-Chfr protein expressed in these cells (Clone 7) may be functional.

Localization to either decondensing chromosomes in telophase cells or to decondensed chromatin in interphase cells helps to confirm Chfr function. **Figure 3-10c** shows Chfr accumulating in the interphase nucleus, with Chfr promoting decondensation. By accumulation of Chfr in telophase, helps to confirm the potential decondensing activity that this protein could have in the EP Chfr checkpoint.

In control cells expressing only EGFP (Clone 4), the EGFP protein localizes throughout the cell, and not specifically to interphase or telophase DNA **(Figure 3-10c, 3-10d**). Cells lacking EGFP-Chfr protein expression are abnormally elongated and have abnormally elongated chromosomes in mitosis indicating a defect either in the G2/M checkpoint or the spindle assembly checkpoint.

3.4.3 SAOS2 and U2OS cells. The mitotic index (MI) assay and the early prophase index (EPI) assay had previously been used with success to detect the presence of Chfr checkpoint activity in SAOS2 and U2OS cells. **Figure 3-11** demonstrates the difference between the mitotic index assay and the early prophase index assay. U2OS cells, which express mutant Chfr, were shown to have a high MI following

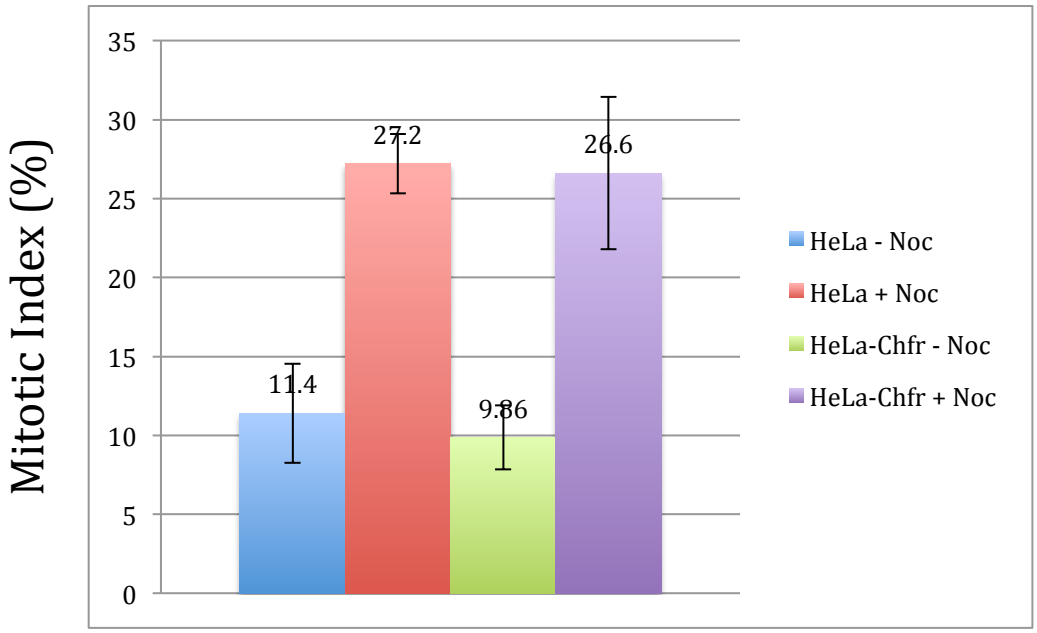


Figure 3-9a. Effect of nocodazole on the mitotic index of unsynchronized HeLa and HeLa-Chfr cells. HeLa and HeLa-Chfr cells were treated with 0.5 µg/ml nocodazole for 9 hours. Mitotic index was counted using phase contrast, where the rounded cells were considered to be arrested at the metaphase-to-anaphase transition in the middle of mitosis.

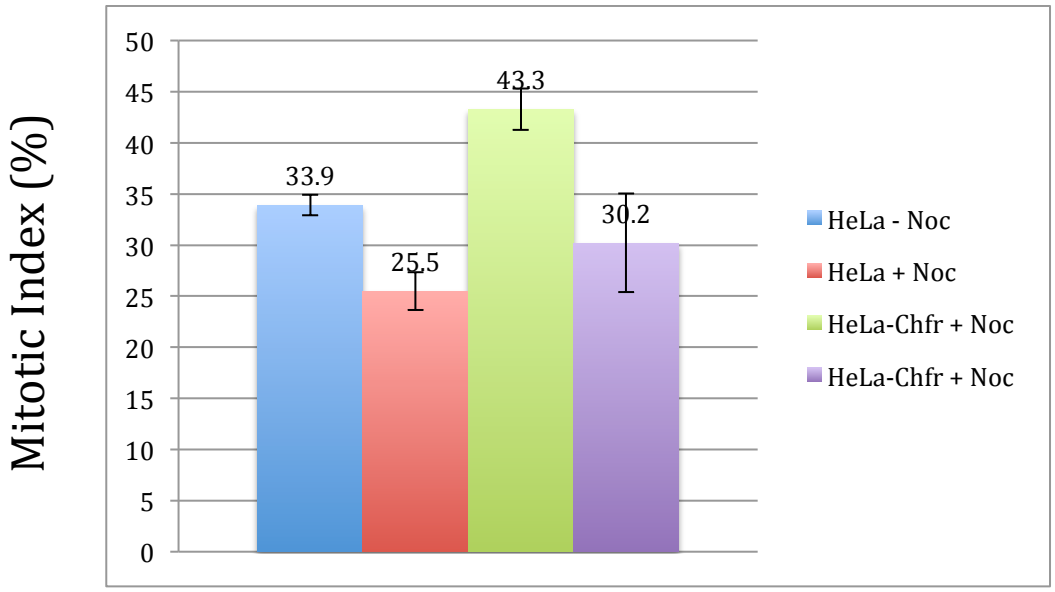


Figure 3-9b. Effect of nocodazole on the mitotic index of G2/M synchronized HeLa and HeLa-Chfr cells. HeLa-CHFR cells were synchronized by a double thymidine, single R03306 block. Cells were treated with 2 mM thymidine for 18 hours, released for 9 hrs, and treated again for 17 hours with 2 mM thymidine to block cells at the G1/S stage of the cell cycle. Thymidine was then removed and R03306, a Cdk1-inhibitor, was used to block cells at the G2/M phase of the cell cycle for 18 hours. Cells were then released from the G2/M block for 45 minutes into nocodazole-containing media, and the mitotic index was scored by counting the number of rounded cells in the cell population.

microtubule damage, indicating reduced Chfr checkpoint activity, whereas SAOS2 cells, which express wild type Chfr were shown to have a lower MI following microtubule damage, indicating Chfr checkpoint activity presence**.** This experiment was done only once.

3.4.4 U2OS-WTChfr and U2OS-RINGΔChfr cells. U2OS WTChfr and U2OS-RINGΔChfr cell lines have been shown by Matsusaka and Pines (2004) to contain and not to contain the Chfr checkpoint, respectively. The two cell lines are isogenic, unlike the SAOS2 and U2OS cell lines. Their tendency to enter mitosis is shown in (**Figure 3-15 and Table 3-1)**.

3.4.4.1 A G2/M checkpoint induced by colcemid in U2OS cells. U2OS cells expressing wild type Chfr (U2OS-WTChfr) and RING finger mutant Chfr (U2OS-RINGΔChfr) cells can express the Chfr protein from a tetracycline-inducible promotor (Matsusaka and Pines, 2004). These proteins are tagged with a green fluorescent X-press tag. WTChfr expression in U2OS cells was confirmed by Western blot **(Figure 3-12).** Immunocytochemistry showed wild type Chfr expression in U2OS cells using the Xpress antibody and the Chfr-CR antibody. Both antibodies localized to spotted areas, some of which could possibly be centrosomes, which are indicated by arrows. The spotted areas are mainly in the interphase nucleus. Approximately 2-10% of cells express WTChfr in the U2OS-WTChfr cell population and approximately 30-40% express RINGΔChfr in the U2OS-RINGΔChfr cell population via immunocytochemistry. This makes their analysis more difficult. The

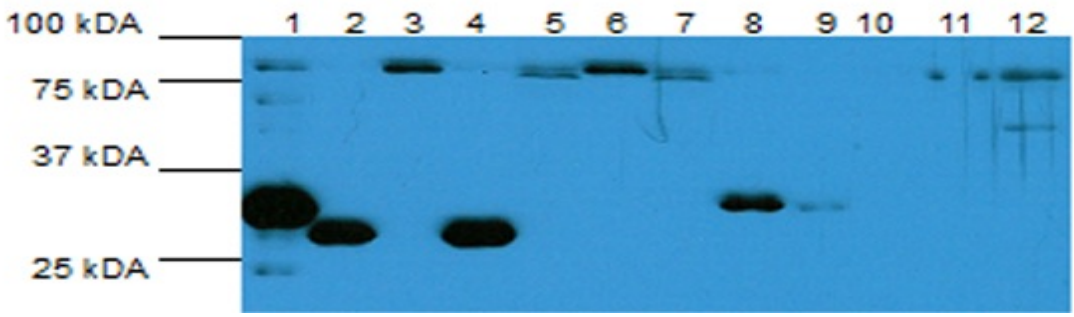


Figure 3-10a. Western blot showing U2OS-EGFP-Chfr clones (lanes 2-12) and the U2OS-GFP control (lane 1). Lanes 2-12, Clones 11-1. Lane 6, for example, contains U20S cell extracts expressing the EGFP-Chfr clone 7 protein, and has high EGFP-Chfr expression in comparison to lane 5, which contains U2OS cells extracts expressing EGFP-Chfr protein clone 8. Clone 8 has medium EGFP-Chfr expression. Clone 3, in lane 10 has even less EGFP-Chfr expression than clone 7 and clone 8. Clone 4, in lane 9, expresses only EGFP and is localized to the nucleus as well as to the cytoplasm.

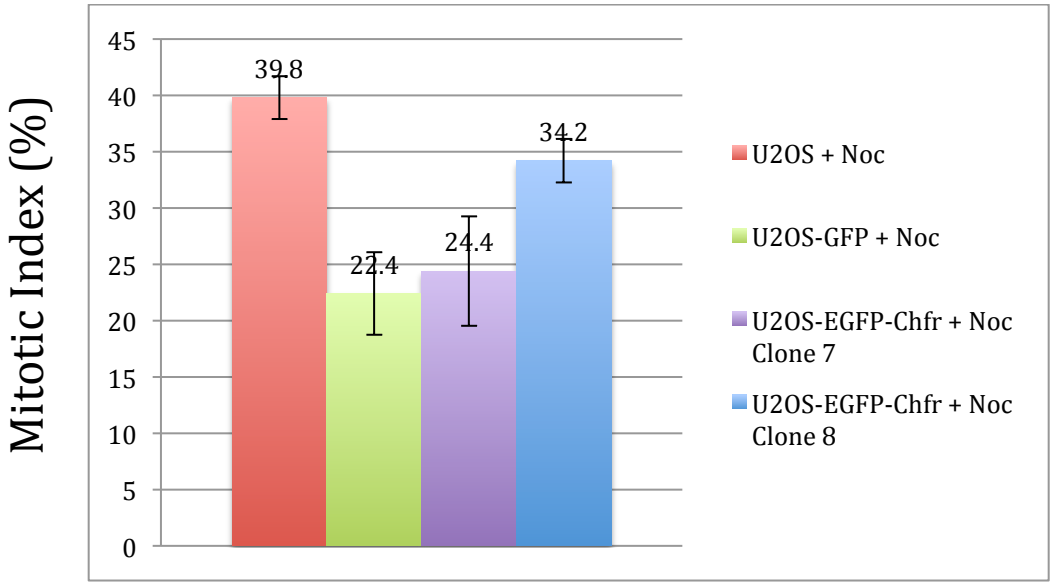


Figure 3-10b. Mitotic index of U2OS EGFP-Chfr clones following nocodazole treatment. U2OS, U2OS-EGFP control cells, and U2OS-EGFP-Chfr cells stably expressing EGFP-Chfr were treated with 0.5 µg/ml nocodazole for 15 hours.

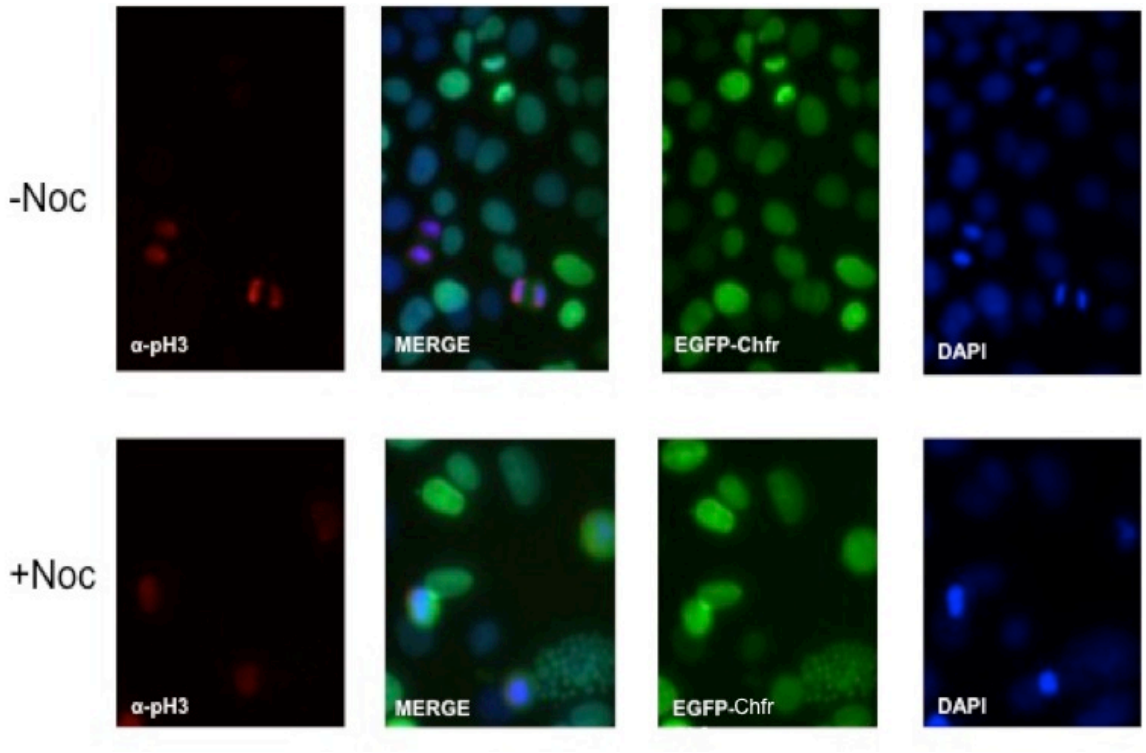


Figure 3-10c. Expression of EGFP-Chfr in U2OS cells. Many cells are rounded in the presence of nocodazole. Arrows point to rounded cells. EGFP-Chfr is either cytoplasmic or localizes to the nucleus. EGFP-Chfr is noticeable in decondensing chromatin in telophase cells. EGFP-Chfr is also present in interphase nuclei with decondensed chromatin. Unlike the EGFP control cell line, this cell line could be more resistant to nocodazole and less prone to cell death because it has less abnormally elongated and fragmented cells.

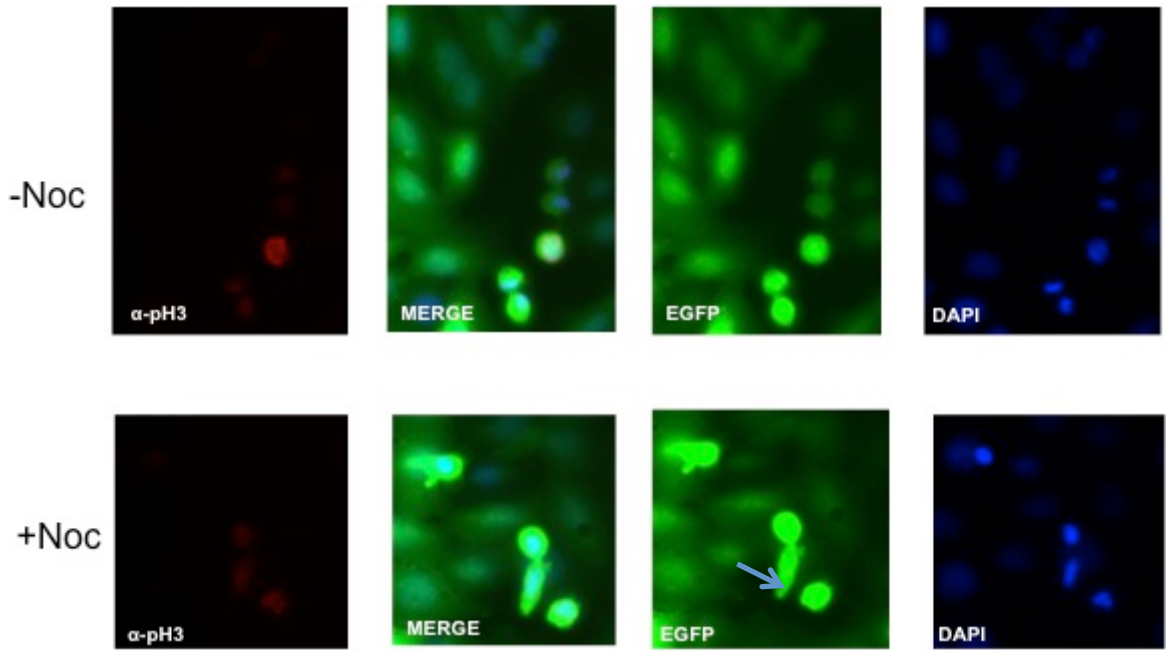


Figure 3-10d. Expression of EGFP in U2OS cells. Many U2OS cells (Clone 4) expressing only the EGFP protein are abnormally elongated and fragmented in nocodazole presence. Arrow points to abnormally elongated cell. The EGFP protein is cytoplasmic.

cells will need to be cloned out to produce homogeneous cell populations. I have synchronized U2OS-WTChfr cells and observed their response to colcemid treatment using phase contrast microscopy. The cells delay their entry into mitosis upon colcemid treatment, and then re-enter mitosis after several hours (Matsusaka and Pines, 2004). The results from this experiment indicate that the cells have a G2/M checkpoint induced by colcemid, but whether Chfr is involved in this checkpoint is still questionable because uninduced U2OS-WTChfr cells also exhibit this delay. The cell line needs to be cloned out to achieve 100% WTChfr expression (instead of 2-10%).

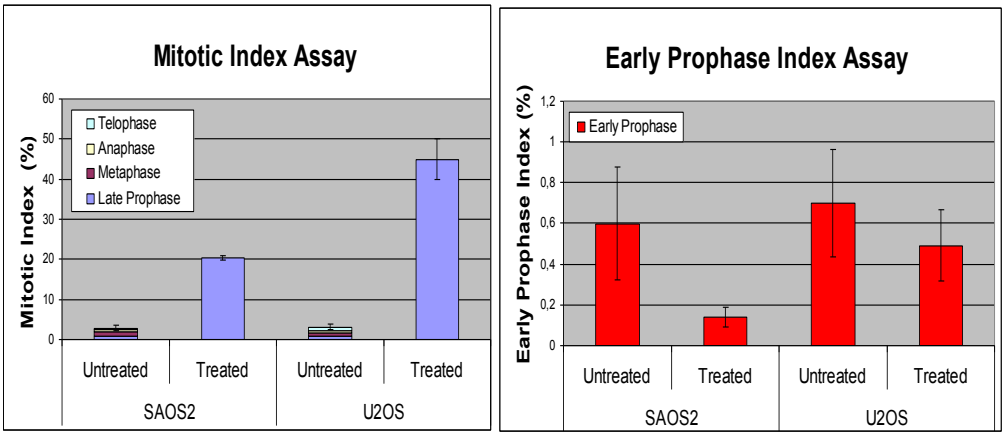
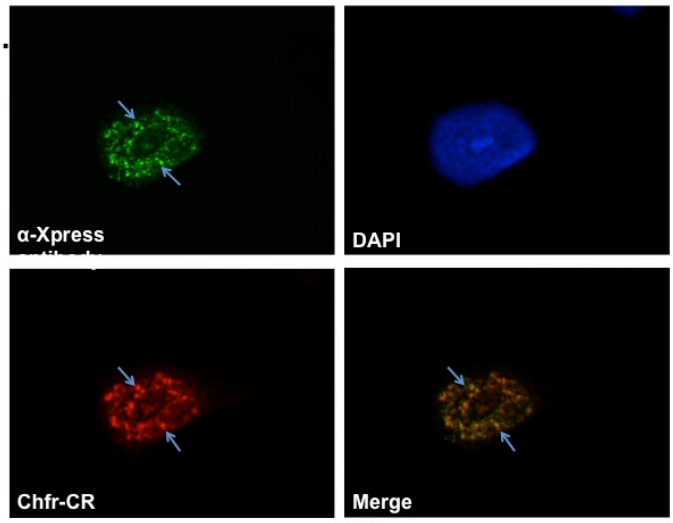


Figure 3-11. A comparison of the current MI assay and the EPI assay tailored for Chfr checkpoint analysis. SAOS2 cell express wild type Chfr and U2OS cells express a mutant version of Chfr. Cells were either untreated or treated with nocodazole.

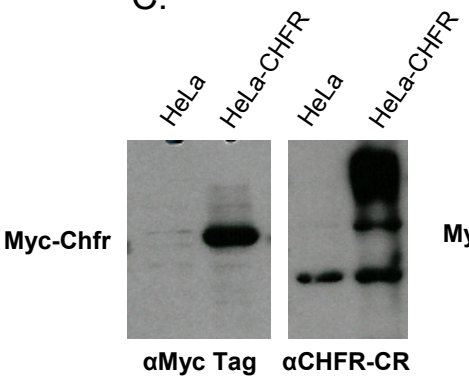
A.

1 = Marker
2 = 9 hrs after removal of tetracycline.
3 = 6 hrs after removal of tetracycline.
4 = 3 hrs after removal of tetracycline.
5 = 0 hrs after removal of tetracycline.
6 = Marker
7 = 9hrs after removal of tetracycline.
8 = 6 hrs after removal of tetracycline.
9 = 3 hrs after removal of tetracycline.
10 = 0 hrs after removal of tetracycline.

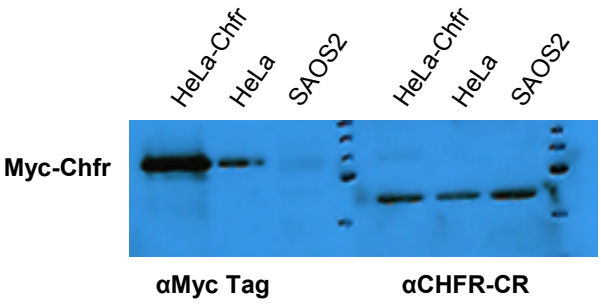
B.



C.



D.



E.

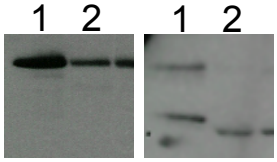


Figure 3-12. (A) Induction of Xpress-WTChfr protein from a tetracycline-inducible promotor in U2OS cells. The Xpress-WTChfr protein was induced for 0, 3, 6, and 9 hours by removal of tetracycline. The Western blot was probed with the anti-Xpress antibody to detect Chfr**. (B-E) The specificity of the Chfr-CR antibody.** Immunocytochemistry showing a cell expressing X-press-WTChfr protein using the anti-Xpress antibody and the Chfr-CR antibody. The Chfr-CR antibody reacts with the same spotted areas as the anti-Xpress antibody indicating that it is Chfr-specific. (C) Myc-Chfr detection using the anti-Myc-tag antibody and the anti-Chfr-CR antibody in HeLa and HeLa-Chfr cells. (D) Detecting Myc-Chfr using the anti-Myc-tag antibody and the anti-Chfr-CR antibody to detect Chfr in HeLa, HeLa-Chfr, and SAOS2 cells. (E) Cells in lane 1 were synchronized with nocodazole overnight. Cells in lane 2 were synchronized with RO3306, treated for 2 hours with nocodazole, and then released for 30 minutes into mitosis.

3.4.4.2 Effect of Chfr expression on mitotic index. Two more similar experiments were carried out using immunocytochemistry instead of phase contrast **(Figures 3-13 and 3-14).** U2OS-WTChfr cells were compared to cells that express a mutant version of the same protein (U2OS-RINGΔChfr). U2OS-WTChfr cells have the following mitotic indexes: 27.1% in uninduced, untreated cells, 8.9% in uninduced, treated cells, 18.5% in induced, untreated cells, and 5.5% in induced, treated cells. Uninduced cells treated with colcemid reduced their MI by 3.0%, whereas induced cells treated with colcemid reduced their MI by 3.4%. This suggests that Chfr expression does not significantly affect the ability of this cell population to delay mitotic entry. However, a complete conclusion cannot be made because of low overall WTChfr expression (~2-10%). There is a colcemid-induced checkpoint in these cells, but WTChfr does not seem to affect the response of cells to colcemid.

Instead of comparing the effect of WTChfr expression to U2OS cells that have not yet been induced to express the WTChfr protein, cells expressing WT-Chfr were then compared to cells expressing a mutant version of the same protein. U2OS-RINGΔChfr cells have the following mitotic indexes: 18.3% for uninduced, untreated cells, 5.1% for uninduced treated cells, (18.3%; 5.1%) 23.2% for induced, untreated cells, and 11.8% for induced, treated cells (23.2%; 11.8%). Uninduced cells treated with colcemid reduced their MI by 3.6 folds, whereas induced cells treated with colcemid reduced their MI by 2.0 folds. We found that cells expressing the mutant version of the protein were less effective at reducing their mitotic index and less efficient at mitotic delay in response to colcemid. These numbers could become more significant with a homogeneous clone.

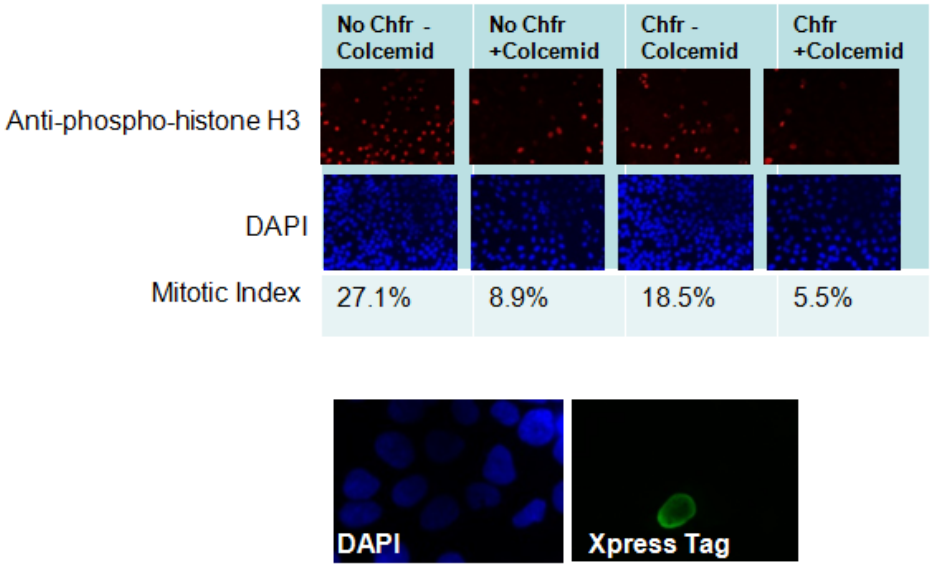


Figure 3-13. Effects of colcemid on the mitotic index of uninduced and induced U2OS-WTChfr cells. Cells were synchronized with RO3306 at the G2/M entry gate, treated or left untreated for 2 hours, and then released into mitosis to observe early mitotic progression. Only approximately 2-10 % of cells in the U2OS-WTChfr cell population express Xpress-WTChfr (lower panel), although some may have low or visually undetectable Chfr expression. Chfr expression affects mitotic index by reducing mitotic index. For example, in the absence of colcemid, the mitotic index of cells is reduced from 27.1% to 18.5% when tetracycline is removed from the cell population to induce Chfr expression. This indicates that expression of Chfr affects entry into mitosis. This is in agreement with some of the published findings. The response of the cell population to colcemid does not seem to be greatly affected by Chfr expression: cells that have no Chfr induction (27.1%; 8.9%) reduce their mitotic index by approximately the same amount than cells that have Chfr induced (18.5%; 5.5%).

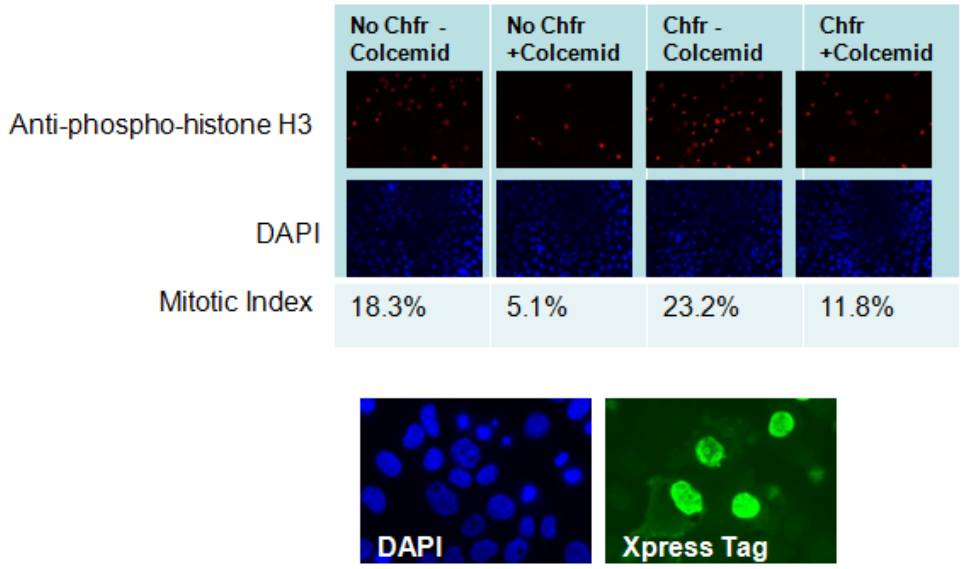


Figure 3-14. Effect of colcemid on the mitotic index of uninduced and induced U2OS-RINGΔChfr cells. Cells were synchronized with RO3306 at the G2/M entry gate, treated or left untreated for 2 hours, and then released into mitosis to observe early mitotic progression. Only approximately 30-40% of cells in the U2OS-RINGΔChfr cell population express Xpress-RINGΔChfr (lower panel). RINGΔChfr expression affects mitotic index by increasing mitotic index.

3.4.4.3 WT-Chfr over-expression prevents mitotic entry. Since the cell populations are not homogeneous, individual green cells expressing the appropriate protein in the induced cell population were observed upon entry into mitosis **(Figure 3-15 and Table 3-1).** U2OS-WTChfr cells have reduced entry into mitosis in comparison to U2OS-RINGΔChfr cells regardless of colcemid presence or absence **(Table 3-1).** The data indicates that cells expressing wild type Chfr are less likely to enter mitosis than are cells expressing mutant Chfr. Cells expressing mutant Chfr lacking the ubiquitinating domain progress into mitosis more readily, possibly because they are unable to carry out the decondensing function. This indicates a mitotic function for the U2OS-WT-Chfr protein. It also indicates the function of the portion of Chfr required for ubiquitination, indicating that ubiquitination is important at the G2/M transition. Aside from the Chfr RING domain mutant control, Ubc13 or Chfr knockdown would also serve as an efficient control to confirm Chfr checkpoint activity in this cell line.

3.5 <u>Ubc13 levels in the early prophase stage of mitosis.</u> HeLa cells expressing Myc-tagged Chfr (HeLa-Chfr; **Figure 3-16a**) were synchronized with the R03306 reagent, which blocks cells at the G2/M entry gate. The cells were then either left untreated or treated with nocodazole for 2 hours before release from the G2/M block. The cells were released for 0, 15, or 40 minutes into mitosis **(Figure 3-16).**

Chfr levels did not change significantly as cells entered mitosis, and a 2 hour

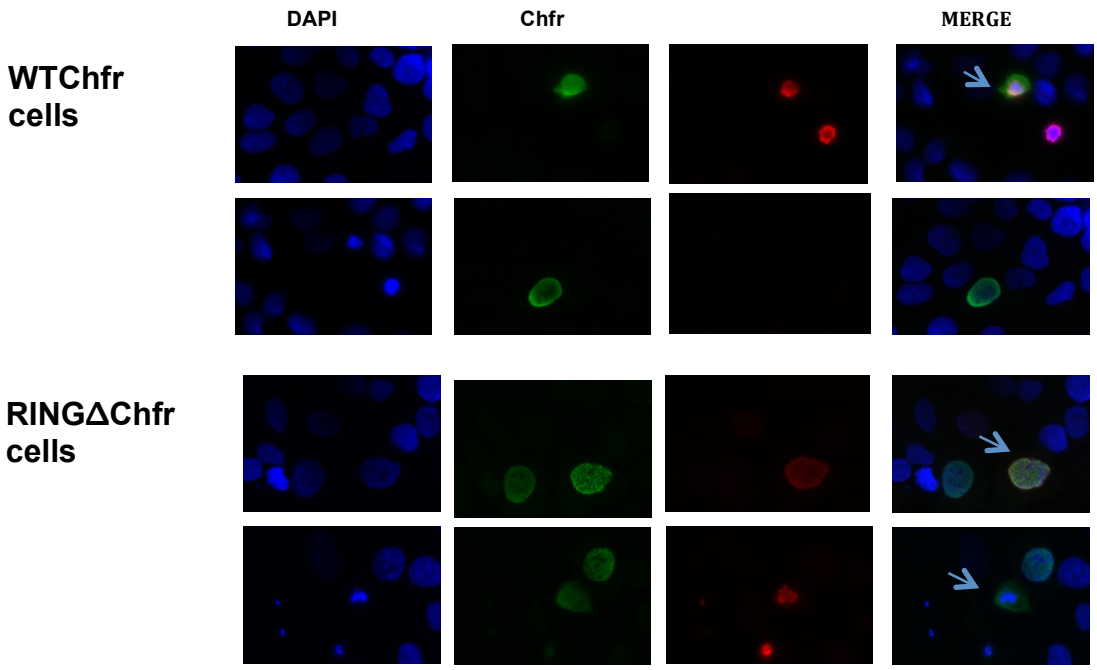


Figure 3-15. The effect of WTChfr and RINGΔChfr protein expression on entry into mitosis in U2OS cells. The anti-Xpress antibody was used to detect wild type and mutant Chfr (green). The phospho-histone H3 (anti-pH3 antibody was used to detect cells that have entered mitosis (red). There is more mitotic cells in the RINGΔChfr–expressing cell population than there are mitotic cells in the U2OS-WTChfr population. These observations may change but indicate that over-expression of WTChfr decreases mitotic entry whereas RINGΔChfr protein expression increases mitotic entry. WTChfr-expressing cells are less likely to contain pH3. This also indicates that the RING domain of Chfr, which is required for the poly-ubiquitination of target proteins, may be required for efficient delay at the G2/M checkpoint. Arrows point to Chfr-expressing, mitotic cells.

Table 3-1. Effects of Chfr protein on cell entry of U2OS cells

	-Colcemid	
U2OS-WTChfr	**0/80**	**0.0%**
U2OS-RINGΔChfr	**10/213**	**4.7%**

	-Colcemid	
U2OS-WTChfr	**0/78**	**0.0%**
U2OS-RINGΔChfr	**14/138**	**10.1%**

Effect of Chfr protein on cell entry in colcemid presence:

	+Colcemid	
U2OS-WTChfr	**0/49**	**0.0%**
U2OS-RINGΔChfr	**1/239**	**0.42%**

	+Colcemid	
U2OS-WTChfr	**1/81**	**1.2%**
U2OS-RINGΔChfr	**4/90**	**4.4%**

This table showing the effect of the wild type Chfr protein on the mitotic entry of U2OS cells in the presence or absence of colcemid. Cells were RO3306-synchronized at the G2/M gate, and treated with colcemid for 2 hours before release into mitosis. Colcemid has a beneficial effect on cells expressing RINGΔChfr, as it reduces the mitotic index of the cell population. Colcemid has very little effect on the mitotic index of the wild Chfr cell population, most possibly because cells expressing the wild type Chfr protein have not yet entered mitosis. Cells expressing the wild type Chfr protein enter mitosis less frequently than RINGΔChfr protein expressing cells, regardless of colcemid presence or absence.

nocodazole treatment did not affect this pattern. Ubc13 levels, on the other hand, *increased* as cells entered into early prophase. This pattern was also not affected by nocodazole treatment. A similar experiment was also carried out using a 2xthymidine-1xRO3306 block and similar results were observed, where Ubc13 protein levels increased within 45 minutes after release from the G2/M block **(Figure 3-16b).** This indicates a role for Ubc13 in mitosis. The actin control here is a little better than in **Figure 16a. Figure 3-16a is similar to Figure 3-16b** in that Ubc13 is increased upon entry into mitosis. This includes the interphase or early prophase time point, and a 45 minute time point following release from the G2/M block.

3.6 Chfr, Ubc13, Uev1a/Mms2 levels at the G2/M transition.

A mitotic shake-off experiment was carried out in nocodazole-treated and untreated HeLa-Chfr cells to study the relative levels of Chfr, Ubc13, and Mms2/Uev1a. Cells were either treated or left untreated with nocodazole for overnight, and the rounded cells were shaken-off, separating the flat cells from the rounded cells. The rounded cells are cells in the late prophase stage of mitosis (LP cell fraction), whereas the flat cells represent cells that are either in interphase or in early prophase (INT/EP cell fraction). Cells were then lysed, and lysates analyzed by Western blot analysis. Ubc13, Uev1/Mms2, and Chfr protein levels were compared

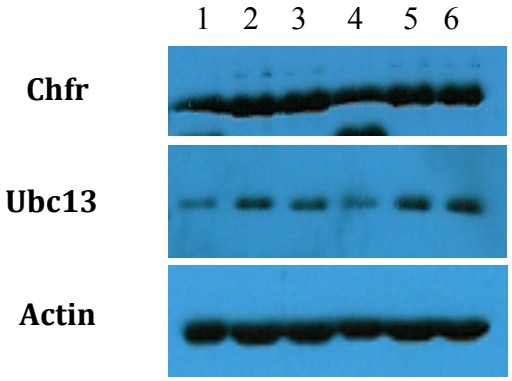


Figure 3-16a. Ubc13 levels upon release from the single RO3306 block. HeLa-Chfr cells were treated with 10 μM of RO3306 for 24 hours. 0.5 μg/ml of nocodazole was added for 2 hours before release from the RO3306-induced G2/M block for various times. A Western blot was carried out to look at the levels of Ubc13, Myc-Chfr, and actin levels upon release into mitosis. The mouse 4E11 Ubc13-specific antibody was used to detect Ubc13 levels, and a rabbit Myc-tag antibody was used to detect Myc-Chfr levels. Immunocytochemistry was done to complement the Western blot, showing cells before and after release from the G2/M block. Lane 1 = 0 min – nocodazole; Lane 2 = 15 min – noccodazole; Lane 3 = 40 min – nocodazole; Lane 4 = 0 min + nocodazole; Lane 5 = 15 min + nocodazole; Lane 6 = 40 min + nocodazole.

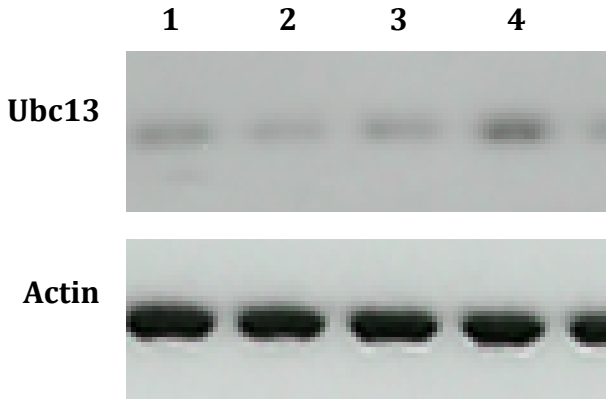


Figure 3-16b. Ubc13 levels upon release from a 2xThymidine-1xRO3306 block. HeLa-Chfr cells were blocked at the G2/M transition and then released into mitosis. The blot was probed with a Ubc13-specific mouse 4E11 antibody. Ubc13 levels rise during the G2/M transition without nocodazole. Lane 1 = 0 minutes release from G2/M block. Lane 2 = 15 minutes release from G2/M block. Lane 3 = 30 minutes release from G2/M block. Lane 4 = 45 minutes release from G2/M block.

in each of the four cell fractions: untreated INT/EP, untreated LP, treated INT/EP, and treated LP **(Figure 3-17).** Ubc13 and Mms2/Uev1 levels were highest in the INT/EP cell fractions and lowest in the LP cell fractions in HeLa-Chfr cells. This pattern was not affected by nocodazole treatment. This, along with the evidence discussed above, supports a role for the Ubc13-Mms2 complex during the interphase or early prophase stage of mitosis, regardless of the presence or absence of microtubule damage.

Chfr levels, on the other hand, were highest in the nocodazole-treated LP cell fractions. This pattern was affected by nocodazole presence, with the Chfr level being *higher* in the nocodazole-treated LP cell fraction and lower in the nocodazole-treated INT/EP cell fraction, the untreated INT/EP cell fraction, and the untreated LP cell fraction. This indicates that Chfr levels increase in the late prophase stage of mitosis only upon extensive nocodazole treatment. The cells in the nocodazole-treated late prophase cell fraction are most likely cells that have been arrested by the spindle checkpoint, which is induced at the metaphase-anaphase transition by microtubule poisons. The more mitotic stress there is, the more Chfr protein is produced by these (HeLa-Chfr) cells. Thus, an increase in Chfr protein levels in this cell fraction indicates a role for Chfr in the spindle checkpoint. There is also a modification of Myc-Chfr in the INT/EP cell fraction, and this modification is completely reduced in the LP cell fraction. This figure indicates that Chfr could possibly auto-ubiquitinate itself using Ubc13 and Mms2/Uev1 at the G2/M transition (**Figure 3-17).**

Figure 3-17 also shows a clear division between the Chfr modification and no Chfr modification at the G2/M and at the LP stage, respectively. Nocodazole does not appear to affect the modification on Chfr. Thus, the shift from Chfr-Ub to Chfr appears to be cell cycle dependent, with the ubiquitin signal serving at the interphase-to-early prophase transition to patrol mitotic entry. **Figure 3-18a** shows similar results. Wherever the nocodazole is highest, Chfr protein level is also highest, to counteract the mitotic stress. Where nocodazole is lowest, Chfr becomes less necessary, and is more likely to become reduced in level through auto-ubiquitination.

Western blot in **Figure 3-18b** further indicates how mitotic stress in the form of nocodazole can modulate such modification in a cell population. In the absence of nocodazole, Chfr is most ubiquitinated. As exposure to mitotic stress increases, the modification on Chfr is reduced and Chfr protein levels increase in a cell population. Under less nocodazole exposure, the Chfr-Ub protein becomes more predominant, because Chfr becomes less necessary under such conditions. Perhaps this modification is a signal for Chfr protein degradation, with Chfr being unnecessary in the absence of microtubule damage.

3.7 The ubiquitin modification on Chfr.

3.7.1 Effect of nocodazole on the Chfr modification. Chfr also appears to be modified in HeLa-Chfr cells, and this modification is reduced as nocodazole treatment time increases. As nocodazole exposure increases, however, nocodazole

addition reduces this polyubiquitin signal to increase Chfr levels, ensuring proper checkpoint control. Unlike Chfr levels, Ubc13, and Mms2/Uev1a levels remain unaffected by increasing doses of nocodazole treatment in HeLa-Chfr cells **(Figure 3-18a).**

The cellular Chfr level, on the other hand, appears to increase upon increasing nocodazole exposure to counteract the mitotic stress **(Figure 3-18a).** The more Chfr there is, the less modification there is on Chfr. **Figure 3-18b** indicates that the observed Chfr modification could be ubiquitination. This experiment was done twice. This indicates a role for Chfr in a response to microtubule damage because the Chfr level increases in response to nocodazole, and is in line with a recently published article (Kim et al., 2011).

Figure 3-18c shows one smear observed in Lane 1 and a very faint band observed in Lane 2. This figure indicates that the modification on Chfr could be K63-linked poly-ubiquitin chains because the smears observed in these lanes coincide with the Chfr modification. Just like the observed modification on Chfr, the potential K63-linked poly-ubiquitin smears decrease with increasing nocodazole exposure in **Figure 3-18a.** Lanes 1-3 contain the HeLa-Chfr whole cell lysate. The experiment was done twice, but the K63-linked poly-ubiquitin smears showed up only in the second blot.

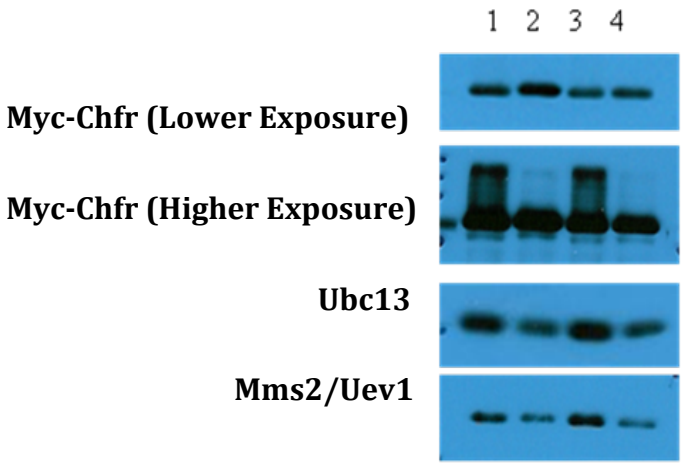


Figure 3-17. Myc-Chfr, Ubc13, and Mms2 protein levels at the G2/M transition in the presence or absence of nocodazole. A mitotic shake-off experiment was carried out in nocodazole-treated and untreated HeLa-Chfr cells. Cells were either treated with or left untreated with nocodazole (for O/N), and the rounded cells were shaken off, separating the flat cells from the rounded cells. Lane 1 = Interphase or early prophase cell fraction + nocodazole (with possible rounded, late prophase like fraction); Lane 2 = Late prophase-like cell fraction + nocodazole; Lane 3 = Interphase or early prophase cell fraction – nocodazole (with possible rounded, late prophase-like cell fraction); Lane 4 = Late prophase-like cell fraction – nocodazole).

3.7.2 The two molecular states of Chfr at G2/M transition. Looking at the state of Chfr in cell populations that are at a particular time point can give a glimpse into how the protein could function at a molecular level. Our data indicates that (a) there are two molecular states of Chfr: Chfr and Chfr-Ub; that (b) Chfr is predominant at LP, whereas, Chfr–Ub is predominant at INT/EP; that (c) Chfr looses its ubiquitin signal after cells pass through the INT/EP transition; that (d) Chfr protein levels clearly increase at LP in response to extensive mitotic stress, after passing through the INT/EP stage; that (e) Chfr, Ubc13, and Mms2/Uev1a levels increase at INT/EP indicating their importance at the G2/M checkpoint gate; and that f) the presence of high Ubc13 and Mms2/Uev1a levels indicates the possibility of K-63 linked poly-ubiquitination at that particular time point. **Figure 3-17** shows that Chfr is modified. **Figure 3-18b and 3-18c** shows that this modification is ubiquitin. The Chfr modification is associated with reduced pH3 levels in the cell population **(Figure 3-18c).**

A molecular mechanism could be envisioned here whereby Chfr auto-ubiquitinates itself using K63-linked poly-ubiquitination. This could temporarily make Chfr stable at the interphase-to-early prophase transition. Chfr-Ub at this stage could then bind to an important early mitotic regulator such as pH3, and using the bulky K63-linked poly-ubiquitin signal interfere with further condensation through stearic hindrance. This could temporarily alter pH3 activity in response to mitotic stress at the early prophase stage of mitosis. K-48-linked poly-ubiquitination could then function to reduce this form of Chfr when no longer necessary, promoting chromosome condensation and progression into mitosis.

3.8 Ubc13 may interact weakly with phosphorylated histone H3 (Figure 3-19a). Ubc13 was pulled out of SAOS2 cells using G-sepharose beads. The blot was probed for Ubc13 using the mouse 4E11 antibody and probed for phosphorylated histone H3 (pH3) using the rabbit anti-phospho-histone H3 antibody. Phosphorylated histone H3 was found to coimmunoprecipitate weakly with Ubc13 indicating an interaction between these two proteins. This experiment was done once. **Figure 3-19b and 3-19c** further support this interaction. These two immunocytochemistry figures support the idea that Ubc13 binds more strongly to mitotic chromosomes in early prophase, metaphase, or telophase cells than to interphase chromatin, although the stronger binding could also be due to nuclear envelope breaking down. Mitotic chromosomes are rich in phosphorylated histone H3. **Figure 3-19b** and **Figure 3-19c** support a potential Ubc13-pH3 interaction, because there is strong immunoreactivity on mitotic chromosomes, and because mitotic chromosomes are rich in phosphorylated histone H3. These experiments, however, would have to be repeated several more times to arrive at a more definite conclusion.

Figure 3-19a, 3-19b, and 3-19c elucidates the possible role of Ubc13 in mitosis in SAOS2 cells, and are in line with **Figure 3-16a**, and **Figure 3-16b,** which show how Ubc13 levels increase upon entry into mitosis. **Figure 3-17** also shows increased Ubc13 levels in the G2/M cell fraction.

One possibility for the function of Ubc13 in mitosis, based on the observation presented, is that Ubc13 serves as a ubiquitin-conjugating enzyme by binding to

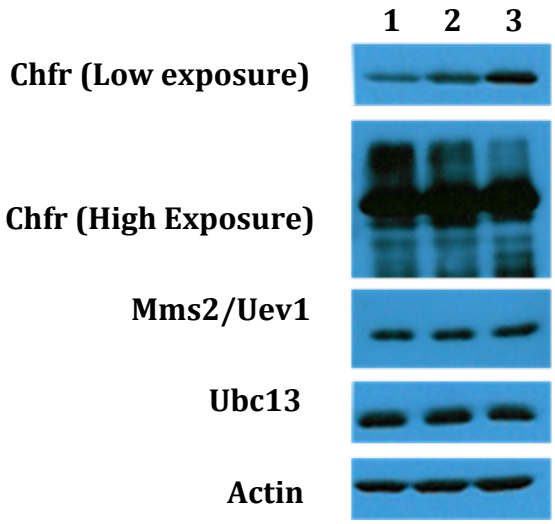


Figure 3-18a. Effects of nocodazole exposure on Myc-Chfr level and modification in HeLa-Chfr cells. HeLa cells expressing Myc-tagged Chfr were treated for 0, 8, and 18 hours with nocodazole and analysed using Western-blotting analysis. Blots were probed with the Myc-tag antibody to visualize Chfr, the 2H11 antibody to visualize Mms2/Uev1, and the 4E11 antibody to visualize Ubc13. Lane 1, 0 hours nocodazole exposure; Lane 2, 8 hours nocodazole exposure.; Lane 3, 18 hours nocodazole exposure.

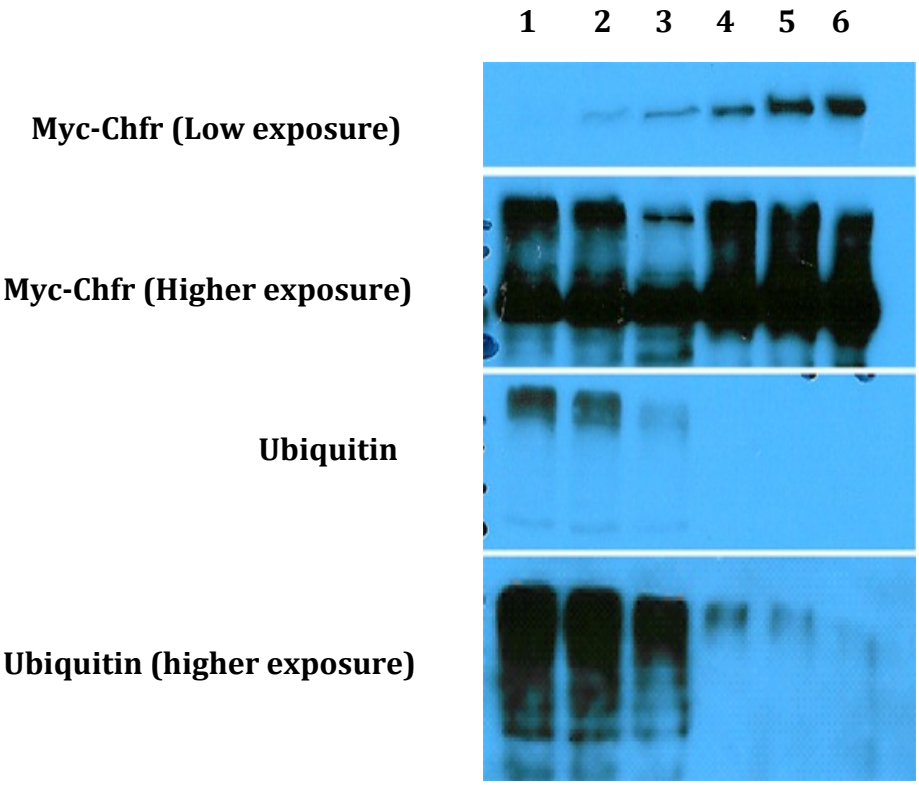


Figure 3-18b. Effects of nocodazole exposure on Myc-Chfr in HeLa-Chfr cells. Extensive treatment of HeLa-Chfr cells with nocodazole results in increased levels of tumor suppressor protein Chfr (lanes 1-3). Lanes 1-3 contain whole cell lysates. Treatment with nocodazole also reduces the amount of modified form of Chfr in the HeLa-Chfr cell population. Chfr was pulled out of whole cell lysate using Myc-tag agarose beads (lanes 4-6). Lanes 4-6 contain immunoprecipitates. The ubiquitin blot overlaps with the Chfr modification indicating that Chfr is modified through ubiquitination.

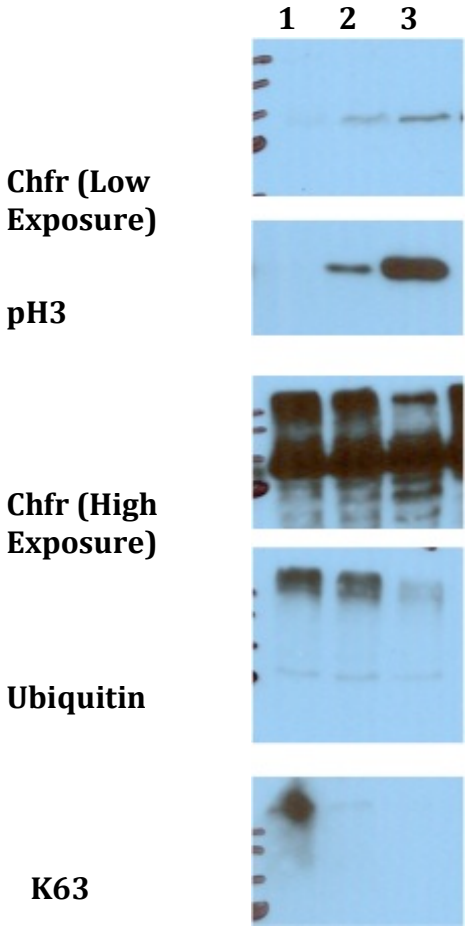


Figure 3-18c. Detecting general ubiquitin and K63-linked poly-ubiquitin chains in HeLa-Chfr whole cell lysates. HeLa-Chfr cells were exposed to nocodazole for 0, 9, and 19 hrs (Lanes 1, 2, and 3), and whole cell lysates were analyzed by Western blot. The blot was probed with a rabbit antibody specific for K63-linked poly-ubiquitin chains (05-1308). As nocodazole increases, Chfr levels increase, pH3 levels increase, the modification on Chfr decreases. The Chfr blot is similar to the ubiquitin and K63-linked poly-ubiquitin chain blot: as nocodazole exposure increases the Chfr modification decreases, the ubiquitin modification decreases, and K63-positive smears decrease. In the K63-linked poly-ubiquitin chain blot, Lanes 1 and 2 have one dark smear and one very faint band, respectively; lane 3 has no band. This figure corresponds to lanes 1, 2, and 3 of Figure 3-17b.

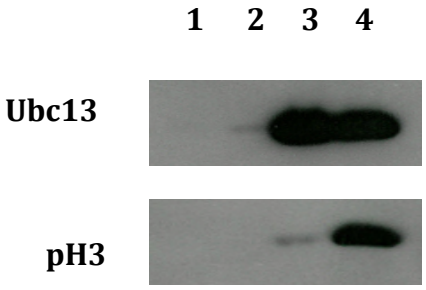


Figure 3-19a. Coimmunoprecipitation of pH3 with Ubc13-specific antibody in SAOS2 cells. Ubc13 interacts weakly with phosphorylated histone H3. Lane 1 = negative control (no whole cell lysate; beads; 4E11 primary antibody); Lane 2 = negative control (whole cell lysate; beads; no 4E11 primary antibody); Lane 3 = CoIP (4E11 primary antibody; whole cell lysate; beads); Lane 4 = whole cell lysate.

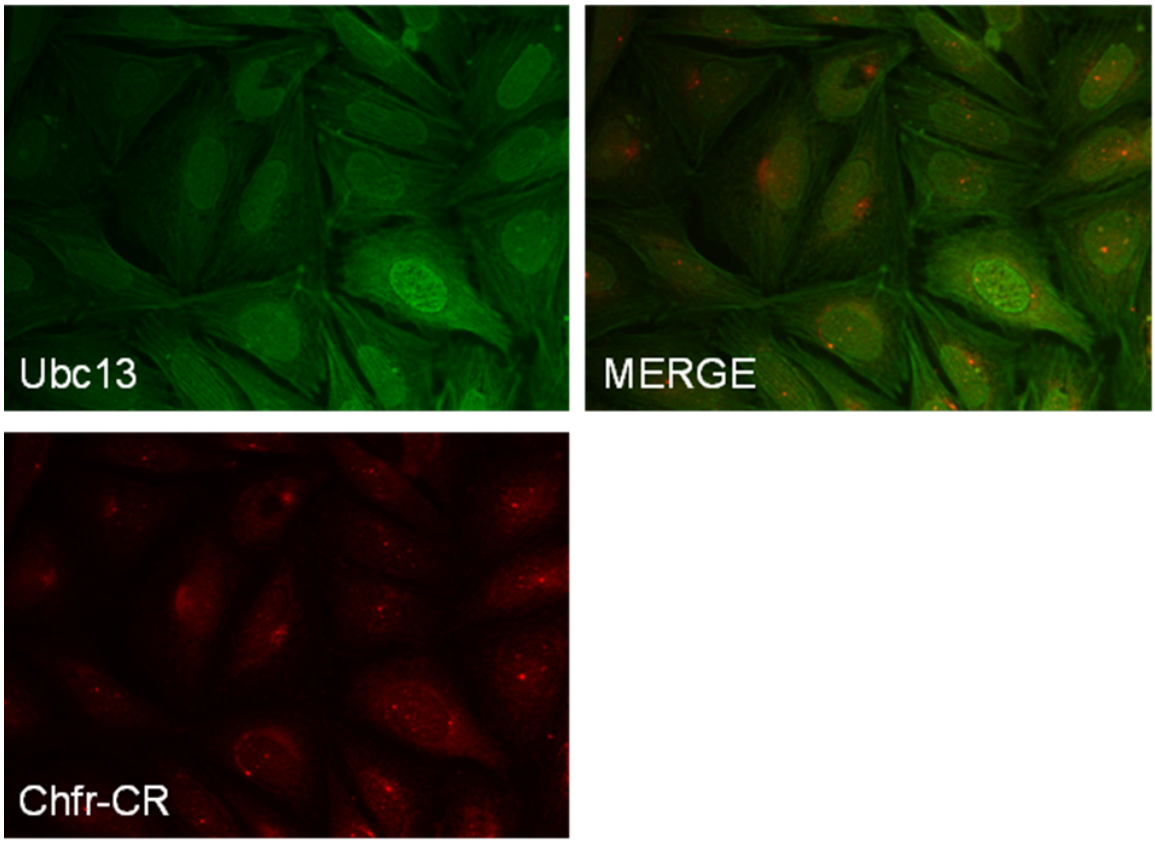


Figure 3-19b. Immunocytochemistry showing SAOS2 cells probed with the Ubc13 antibody and the Chfr-CR antibody. This figure shows an early prophase SAOS2 cell with strong immunoreactivity on the mitotic chromosomes. Chfr localizes to the centrosomes brightly. SAOS2 cells were fixed and permeabilized with -20°C methanol for 5 minutes, and were then incubated with the mouse 4E11 Ubc13-specific antibody and the rabbit Chfr-CR antibody overnight. The DAPI stain was not applied.

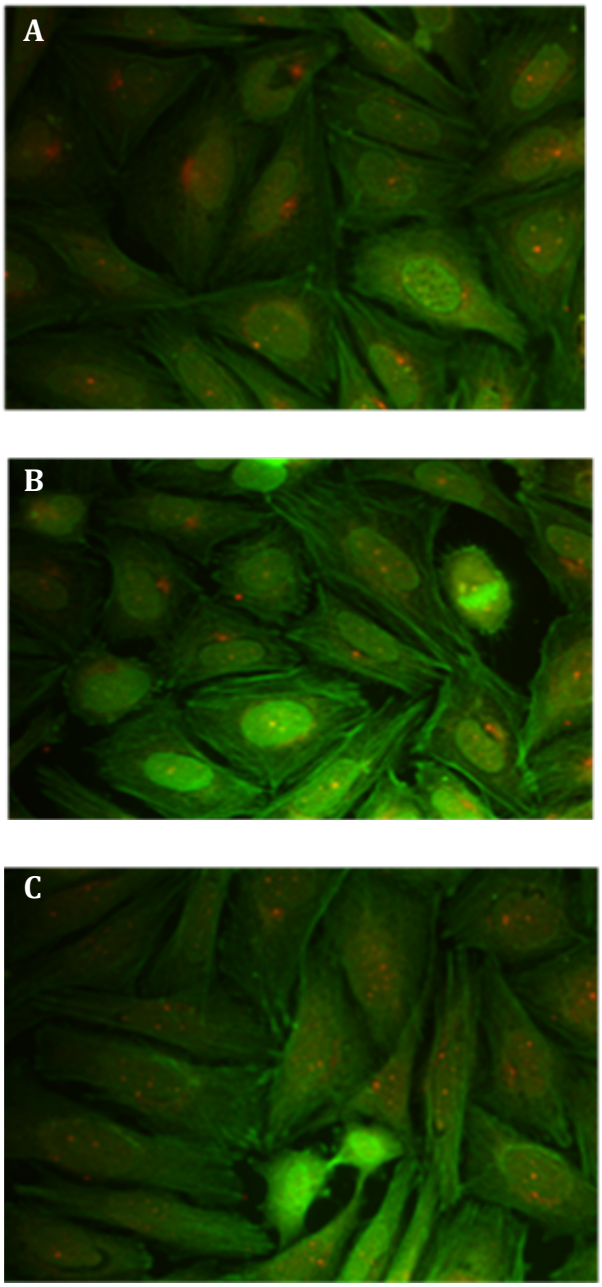


Figure 3-19c. Ubc13 immunoreactivity on mitotic chromosomes. SAOS2 cells were fixed and permeabilized with -20°C methanol for 5 minutes, and were then incubated with the mouse 4E11 Ubc13-specific antibody and the rabbit Chfr-CR antibody overnight. The DAPI stain was not applied. The photos show an (A) early prophase cell, (B) a metaphase cell, and (C) a telophase cell, all of which have phosphorylated histone H3 on chromosomes. Together with appropriate levels of

Chfr, which in this particular cell line appear to be low around the DNA region, mitotic chromosomes have 4E11 immunoreactivity stronger than their surroundings. The rabbit Chfr-CR antibody localizes to the centrosomes.

Chfr and phosphorylated histone H3 on chromosomes. Ubc13 could possibly attach ubiquitin chains onto the chromatin or onto Chfr itself, to signal a conformational change, promoting chromosome condensation.

3.9 Chfr also interacts with phosphorylated histone H3 upon entry into mitosis (Figure 3-20a and Figure 3-20b).

Chfr was pulled out of HeLa-Chfr cells using the anti Myc-tag agarose bead conjugate. The blot was probed for Chfr using the rabbit Myc-tag antibody and probed for phosphorylated histone H3 (pH3) using the rabbit anti-phospho-histone H3 antibody. Lanes 1 and 2 in **Figure 3-20a** show that when Myc-Chfr is present and is pulled out of the whole cell lysate, phosphorylated histone H3 is pulled out as well; but when Chfr is not present in the whole cell lysate, phosphorylated histone H3 does not coimmunoprecipitate. Lanes 3 and 4 indicate equal loading of both HeLa and HeLa-Chfr whole cell lysates to the beads.

A second experiment was done to confirm this interaction, as shown in **Figure 3-20a.** HeLa and HeLa-Chfr whole cell extracts were applied to Myc-tagged agarose beads to coprecipitate Myc-Chfr bound to phosphorylated histone H3 (pH3). Varying amounts of whole cell extract were applied to the beads. Lanes 3 and 4 indicate that endogenous phosphorylated histone H3 coimmunoprecipitates with Myc-Chfr in HeLa-Chfr cells. Lanes 5 and 6 contain HeLa and HeLa-Chfr whole cell

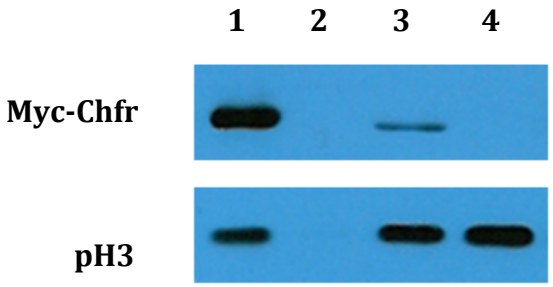


Figure 3-20a. Chfr interacts with phosphorylated histone H3. Chfr was pulled out of HeLa-Chfr cells using the anti-Myc-tag agarose bead conjugate. The blot was probed for Chfr using the rabbit myc-tag antibody and probed for phosphorylated histone H3 (pH3) using the rabbit anti-phospho-histone H3 antibody. Lane 1 = IP HeLa-Chfr; Lane 2 = IP HeLa; Lane 3 = HeLa-Chfr whole cell lysate; Lane 4 = HeLa whole cell lysate.

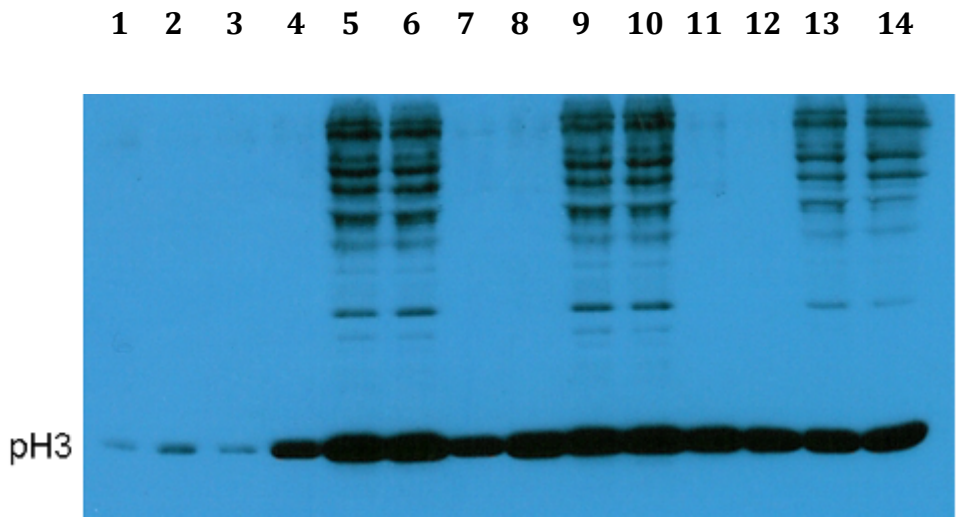


Figure 3-20b. Chfr interaction with phosphorylated histone H3. HeLa and HeLa-Chfr whole cell extracts were applied to Myc-tagged agarose beads to coprecipitate Myc-Chfr bound to phosphorylated histone H3 (pH3). Varying amounts of whole cell extract were applied to the beads: Lanes 1 and 2, 528 µg; Lanes 3 and 4, 1056 µg; Lanes 7 and 8, 2112 µg; Lanes 11 and 12, 3168 µg. Lanes 3 and 4 indicate that endogenous phosphorylated histone H3 coimmunoprecipitates with Myc-Chfr in HeLa-Chfr cells. Lanes 5, 6, 9, 10, 13 and 14 contain HeLa and HeLa-Chfr whole cell lysates to show equal loading. Lanes 3 and 4 were chosen because they most clearly indicate the difference between HeLa and HeLa-Chfr cells with respect to the interaction.

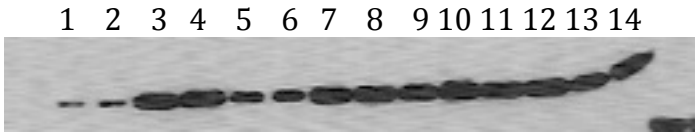


Figure 3-20c. Coimmmunoprecipitation of Chfr and phosphorylated histone H3 in the presence of nocodazole. HeLa and HeLa-Chfr cell lysates were lysed and prepared for Western blot analysis. Various amounts of whole cell lysate were applied to the beads and loaded onto the gel to observe pH3 binding to Chfr at various applied protein concentrations. The anti-phospho-histone H3 antibody was used to detect pH3 (3:10000). The anti-Myc tag antibody was used to detect Chfr (3:10000). Nocodazole treatment also affects the interaction between Chfr and phosphorylated histone H3. When nocodazole is present the amount of pH3 in Lane 2, for example, is the same as the amount of pH3 in Lane 1. This is in contrast to the experiment done in the absence of nocodazole. Lane 1 = HeLa (30 μg protein); 2 = HeLa-Chfr (30 μg protein); 3 = HeLa whole cell lysate; Lane 4 = HeLa-Chfr whole cell lysate; Lane 5 = HeLa (60 μg protein); Lane 6 = HeLa-Chfr (60 μg protein); Lane 7 = HeLa whole cell lysate; Lane 8 = HeLa-Chfr whole cell lysate; Lane 9 = HeLa (120 μg protein); Lane10 = HeLa-Chfr (120 μg protein); Lane 11 = HeLa whole cell lysate; Lane 12 = HeLa-Chfr whole cell lysate; Lane 13 = HeLa (240 μg protein); Lane 14 = HeLa-Chfr (240 μg protein).

lysates to show equal loading. Lanes 3 and 4 were chosen because they most clearly indicate the difference between HeLa and HeLa-Chfr cells with respect to the interaction. Another possible alternative control for the experiment could be HeLa cells containing Myc-tag only. **Figure 3-20c** shows in one experiment that when nocodazole is added the Chfr-pH3 interaction is reduced. This experiment would have to be repeated several more times to arrive at a more definite conclusion. **Figure 3-18c** shows how nocodazole levels affect Chfr and pH3 levels. As nocodazole exposure increases, Chfr protein level increases and pH3 level increases. These two figures helps to point out what happens in chromosomes when Chfr is altered through nocodazole. In the presence of nocodazole, Chfr can no longer bind to pH3, so it cannot affect DNA structure, which is most likely why pH3 levels rise as nocodazole exposure increases.

3.10 Chfr appears to be phosphorylated upon entry into mitosis.

Mitotic shake-off was performed on nocodazole-treated SAOS2 cells. Nocodazole is a microtubule poison. SAOS2 cells were divided into adherent and the non-adherent cell fractions. The adherent cell fraction represents interphase or early prophase cells, whereas the non-adherent cell fraction represents cells in the late prophase stage of mitosis. **Figure 3-21** clearly shows that as cells enter the late prophase stage of mitosis, the single Chfr band turns into a doublet, indicating that Chfr is modified, possibly by phosphorylation, during the late prophase stage of

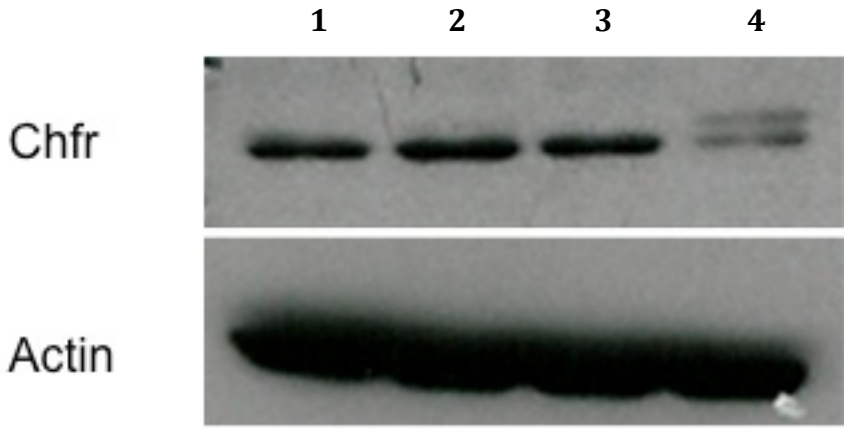


Figure 3-21. Western blot showing that the Chfr protein maybe phosphorylated and its level reduced upon entry into mitosis. Cell fractions were collected by mitotic shake off. Blot was probed with a Chfr rabbit antibody specific for the C-terminus of Chfr. INT/EP = interphase and early prophase cell fraction. LP = late prophase cell fraction. Lane 1 = SAOS2 Untreated; Lane 2 = SAOS2 + Nocodazole; Lane 3 = SAOS2 + Nocodazole INT/EP; Lane 4 = SAOS2 + Nocodazole LP.

mitosis. Furthermore, the levels of Chfr appear to be reduced upon entry into the later stage of mitosis. Together, these findings indicate that Chfr may be phosphorylated. Kim et al. (2011) found that the phosphorylation of Chfr is involved in controlling the auto-ubiquitinating activating of Chfr. When phosphorylation is reduced, this also reduces the auto-ubiquitinating activity of Chfr.

3.11 Localization of Ubc13, EYFP-Mms2, and Chfr to the centrosomes of interphase and mitotic cells.

Interestingly, I have noticed that Ubc13, Mms2, and Chfr localize to the centrosomes in interphase SAOS2 cells and in mitotic SAOS2 cells **(Figure 3-22 and 3-23)**. Centrosomes are structures within cells that serve as microtubule organizing centers. Microtubules are important because they are necessary to pull the DNA matter apart into two equal halves during mitosis – one into each daughter cell. If microtubules are not organized accurately, then mitosis may not occur accurately, resulting in chromosome mis-segregation and genetic instability. Chfr and Ubc13 localize to these structures, just like Aurora A and Plk1 (Summers et al., 2005), which promote progression through mitosis and are two potential Chfr target proteins. Furthermore, Mms2 also appears to be present in the centrosomes**.** Mms2 is a Ubc13 coenzyme necessary for the K-63 linked poly-ubiquitination activity of Ubc13. These observations imply that Chfr, Ubc13, and Mms2 function in the

centrosomes during mitosis to generate K-63-linked polyubiquitin chains, perhaps targeting proteins such as Aurora A or Plk1.

The Ubc13 enzyme alone can potentially catalyze the formation of both K63-linked and K48-linked poly-ubiquitin chains but, when it pairs with Mms2 or Uev1, it generates K63-linked poly-ubiquitin chains exclusively. Thus, it would be worthwhile to distinguish which of the two Uevs colocalizes to the centrosomes with Chfr and Ubc13. If Mms2 or Uev1 or both colocalize to the centrosomes with Chfr, this would be significant as it would further support the idea that Chfr, Ubc13, and Mms2/Uev1 work together during mitosis to signal cellular stress through K63-linked poly-ubiquitination.

Figure 3-22 illustrates Ubc13 and Chfr localizing to the centrosomes in SAOS2 cells via immunocytochemistry. Immunocytochemistry showed that Chfr and Ubc13 localize to the centrosomes in early prophase and late prophase SAOS2 mitotic cells. SAOS2 cells have been shown to contain a functional Chfr checkpoint. Two different immunocytochemistry techniques were used to visualize this localization to small round structures that were either single or in duplicate. The single round structures were probably centrosomes in interphase cells, while the duplicate structures were duplicated centrosomes in cells that were just beginning to enter mitosis or were already in mitosis.

In one experiment, SAOS2 cells were fixed with 4% formaldehyde for 30 minutes and permeabilized with methanol for 5 minutes, after which they were stained with the rabbit CHFR-CR antibody **(Figure 3-22a).** In the second

experiment, SAOS2 cells were fixed simultaneously with 4% formeldahyde and 0.5% NP40 for 15 minutes on ice, after which they were stained with the rabbit CHFR-CR antibody and the mouse 4E11 antibody **(Figure 3-22b).** Ubc13 and Chfr localization was more clearly observed by using the second technique. These experiments indicate that Ubc13 and Chfr localize to the centrosomes of early and late prophase SAOS2 cells, suggesting a role for Ubc13 and Chfr in mitosis. Ubc13 localization to the centrosomes was confirmed by using the gamma tubulin antibody. **Figure 3-22c** shows a cell with Ubc13 in the centrosome region and in the mitotic spindle. At this particular location, Chfr could colocalize to sense microtubule damage, and together with Ubc13/Mms2 generate signal that stops mitosis. A proper Chfr, Ubc13, and Mms2/Uev1 knockdown could further help to confirm this centrosome and mitotic spindle localization. Chfr, Ubc13, and Mms2/Uev1 could localize to the centrosomes to poly-ubiquitinate Aurora A or Plk1 either for destruction to decrease protein level and progression into mitosis, or to alter Aurora A and Plk1 protein function through K63-linked poly-ubiquitination**.**

Mms2 was also shown to localize to the centrosomes by two different techniques **(Figure 3-23).** In one experiment, SAOS2 cells expressing the Mms2 protein fused to an enhanced yellow fluorescent protein (SAOS2 pEYFP-Mms2 cells) were fixed with -20ºC methanol for 5 minutes and were then stained with either the GFP antibody **(Figure 3-23a, 3-23b, 3-23c)** or the mouse 2h11 antibody specific for Mms2/Uev1 **(Figure 23d)** and the rabbit gamma tubulin antibody. EYFP-Mms2 was shown to localize to the centrosomes with the GFP antibody **(Figure 3-23a, 3-23b, 3-23c)**, and also with the 2h11 antibody, though very faintly **(Figure 3-23d).**

In particular, Mms2 was found to distinctly localize to only one of the centrosomes in **Figure 3-23d**, indicating a potential role for this protein in centrosomes duplication.

In another experiment, SAOS2 pEYFP-Mms2 cells were fixed simultaneously with 4% formaldehyde and 0.5% NP40 for 15 minutes and were then stained with the rabbit GFP antibody and the mouse gamma tubulin antibody **(Figure 3-23e).** This second technique showed more clearly EYFP-Mms2 localization to the centrosomes of SAOS2 cells.

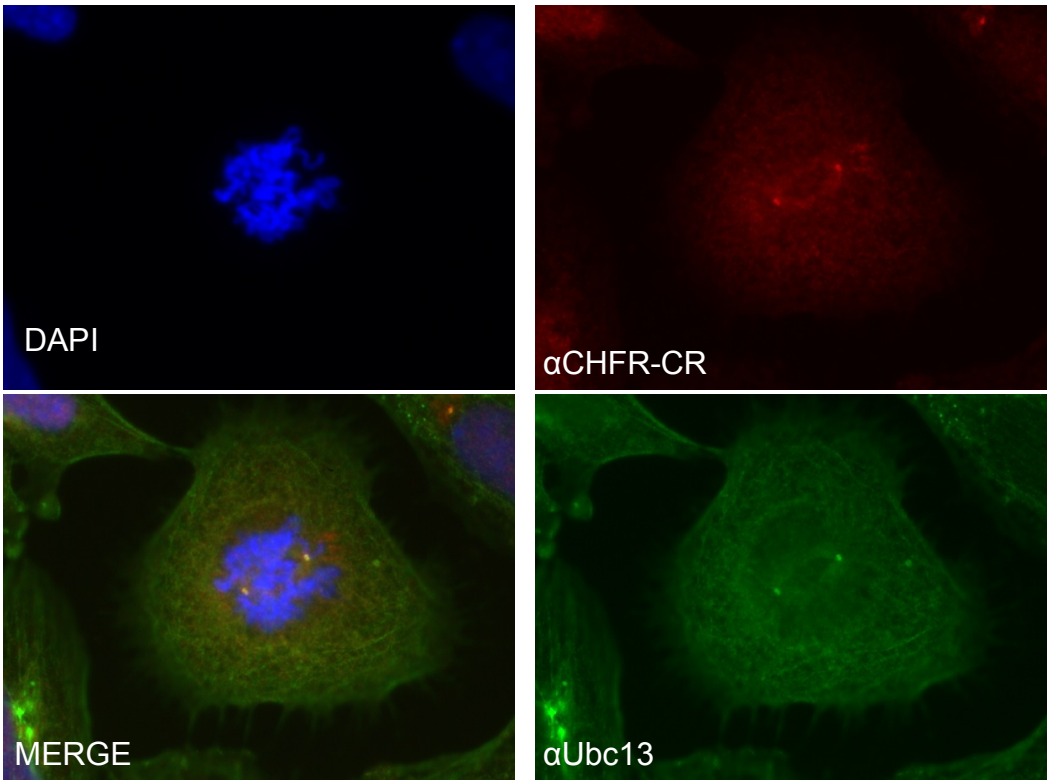


Figure 3-22a. Chfr and Ubc13 appear to localize to the centrosomes in mitotic SAOS2 cells. This figure shows the two proteins of interest localizing to a centrosomes in late prophase. SAOS2 cells were fixed with 4% formaldehyde for 30 minutes and permeabilized with methanol for 5 minutes, after which they were stained with the rabbit CHFR-CR antibody and the mouse Ubc13 antibody.

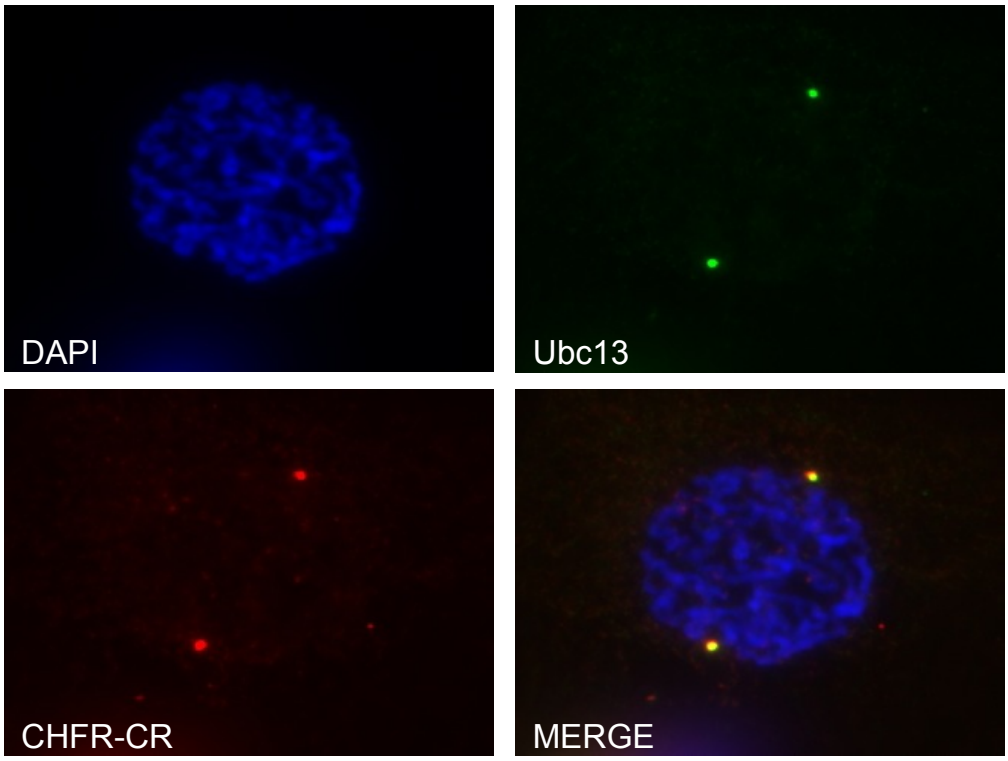


Figure 3-22b. Chfr and Ubc13 appear to localize to the centrosomes in early prophase mitotic SAOS2 cells. SAOS2 cells were fixed simultaneously with 4% formeldahyde and 0.5% NP40 for 15 minutes on ice, after which they were stained with the rabbit CHFR-CR antibody and the mouse 4E11 antibody.

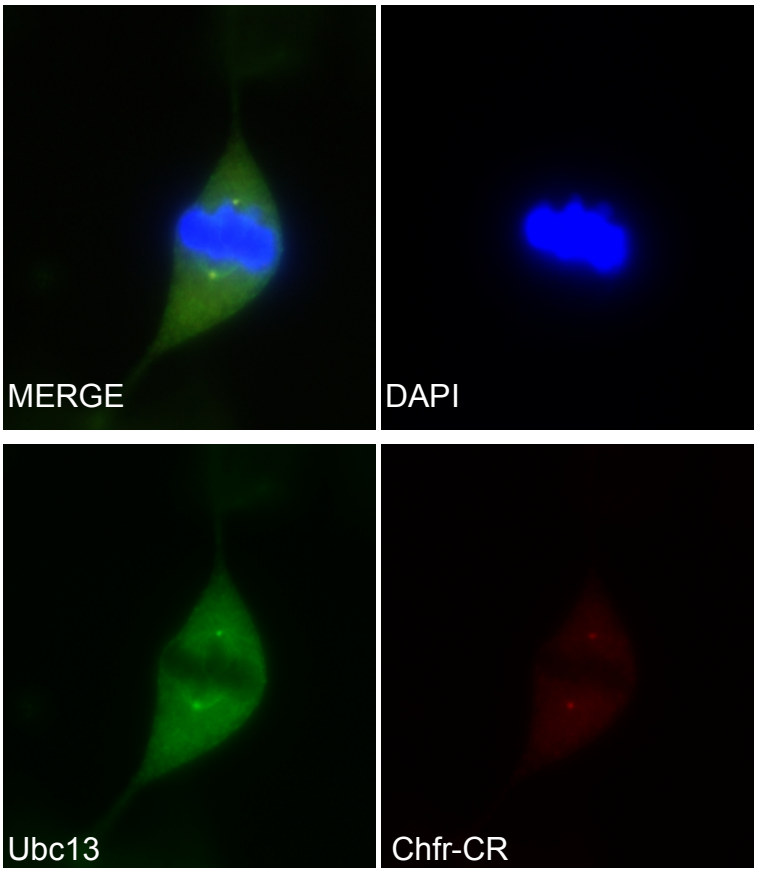


Figure 3-22c. Ubc13 localized with the centrosomes and also to the mitotic spindle in some mitotic cells. SAOS2 cells were fixed with -20°C methanol for 5 minutes and probed with 4E11 Ubc13 specific antibody as well as the Chfr-CR antibody overnight.

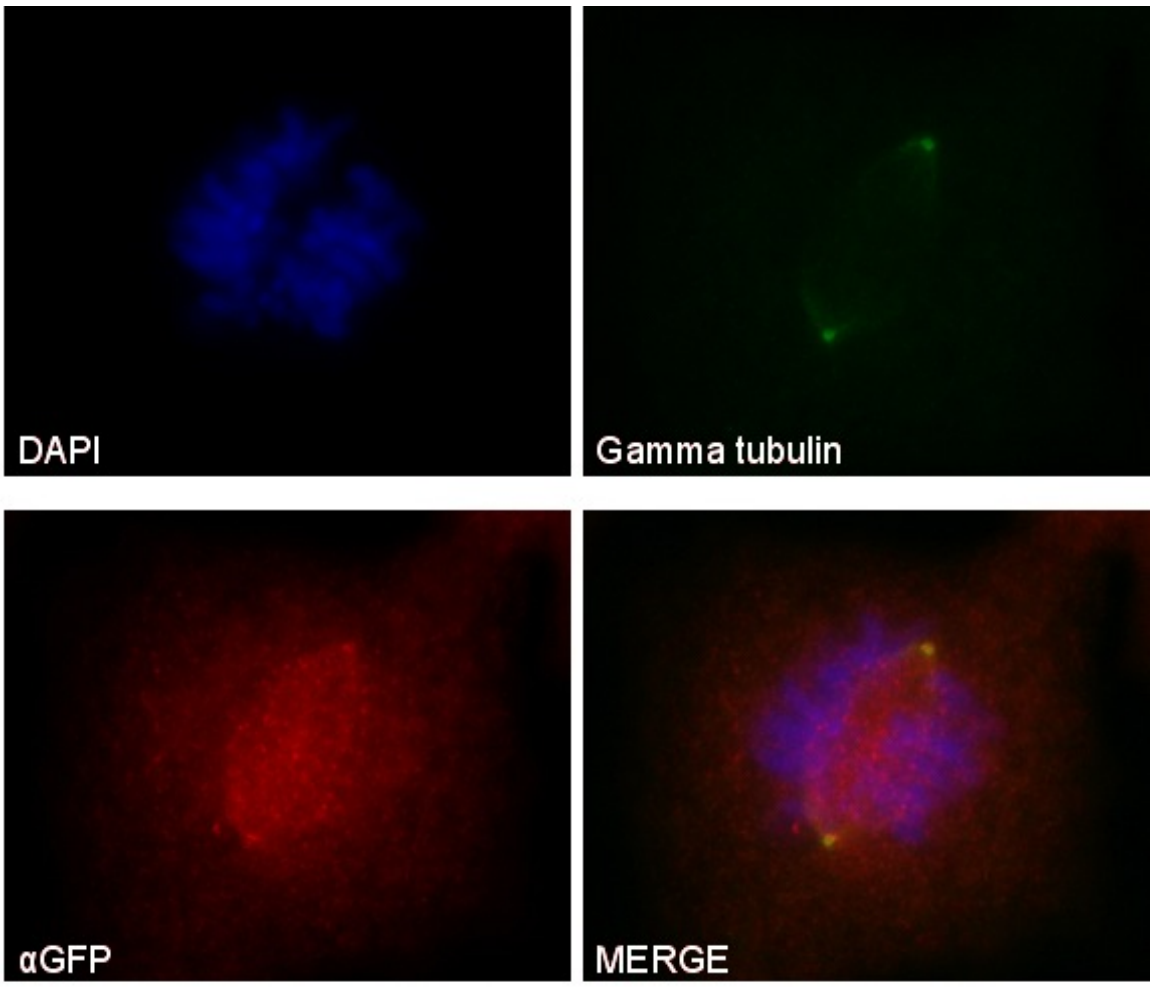


Figure 3-23a. EYFP-Mms2 appears to localize to the mitotic spindle in SAOS2 cells stably expressing EYFP-Mms2. SAOS2 pEYFP-Mms2 cells were fixed with -20°C Methanol on ice for 5 minutes, after which they were stained with the rabbit GFP antibody and the mouse gamma tubulin antibody. In this figure EYFP-Mms2 (rabbit anti-GFP primary antibody) localizes faintly not only to the centrosome region, but also to the mitotic spindle apparatus, which is confirmed by the gamma tubulin stain.

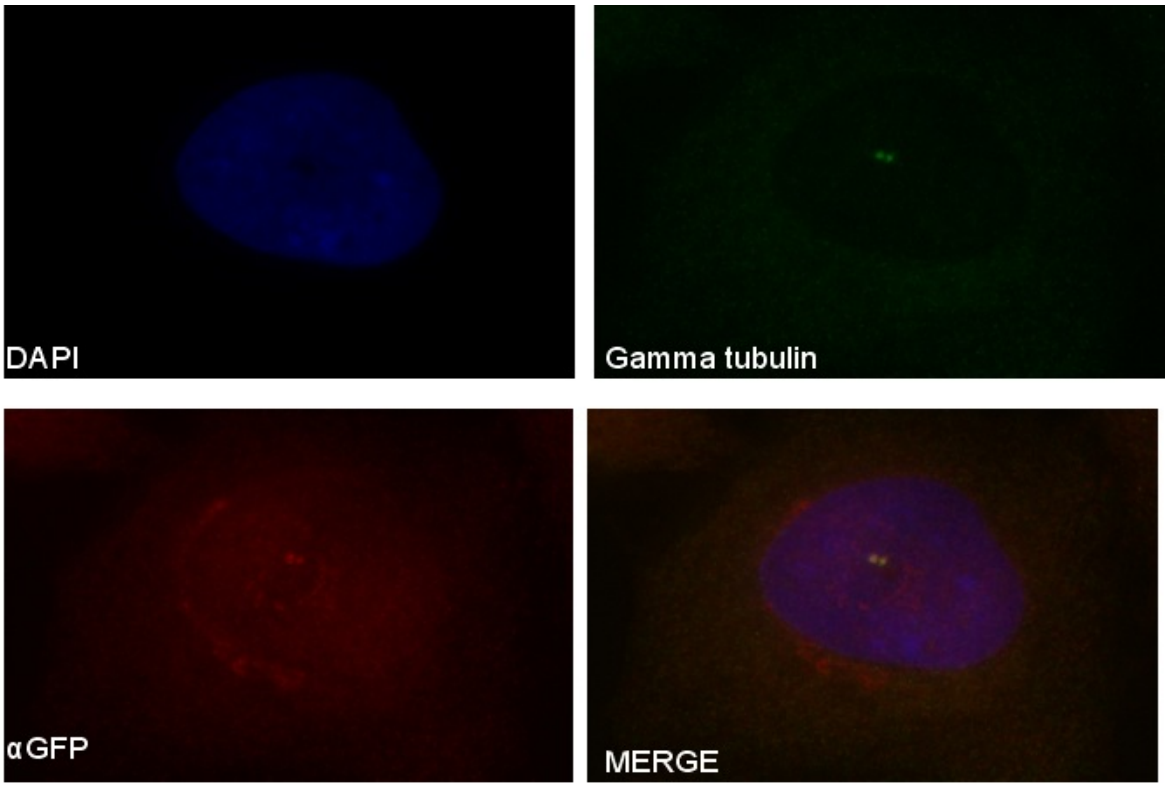


Figure 3-23b. EYFP-Mms2 appears to localize to the mitotic spindle in SAOS2 cells stably expressing EYFP-Mms2. SAOS2 pEYFP-Mms2 cells were fixed with -20°C Methanol on ice for 5 minutes, after which they were stained with the rabbit GFP antibody and the mouse gamma tubulin antibody.

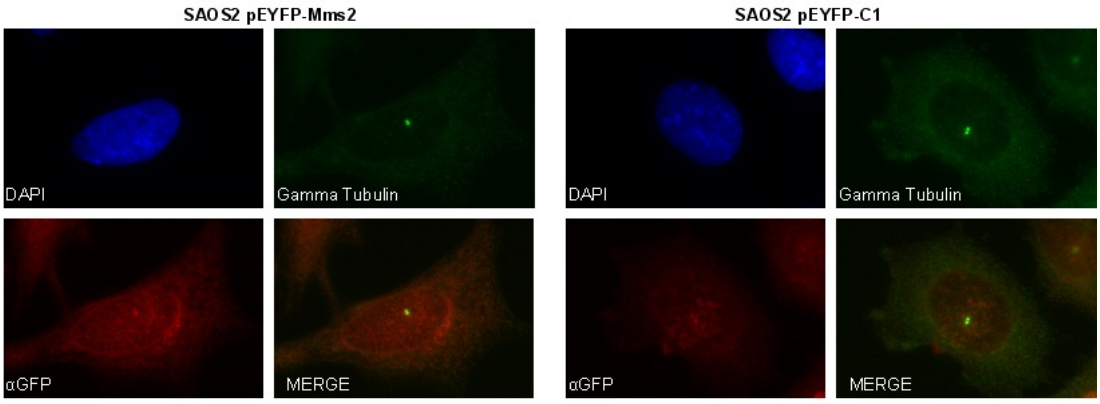


Figure 3-23c. EYFP-Mms2 appears to localize to the centrosomes in SAOS2 cells stably expressing EYFP-Mms2. SAOS2 cells stably expressing pEYFP-Mms2 (A) and SAOS2 cells transiently transfected with the pEYFP-C1 empty control vector (B), were fixed with -20°C Methanol on ice for 5 minutes, after which they were stained with the rabbit GFP antibody and the mouse gamma tubulin antibody. Staining is reduced in SAOS2 cells stably expressing pEYFP-C1. Non-specific binding is visible, but the cells expressing pEYFP-Mms2 have a noticeably brighter stain both in general throughout the cell and also in the centrosome. The gamma tubulin antibody confirms the centrosome localization.

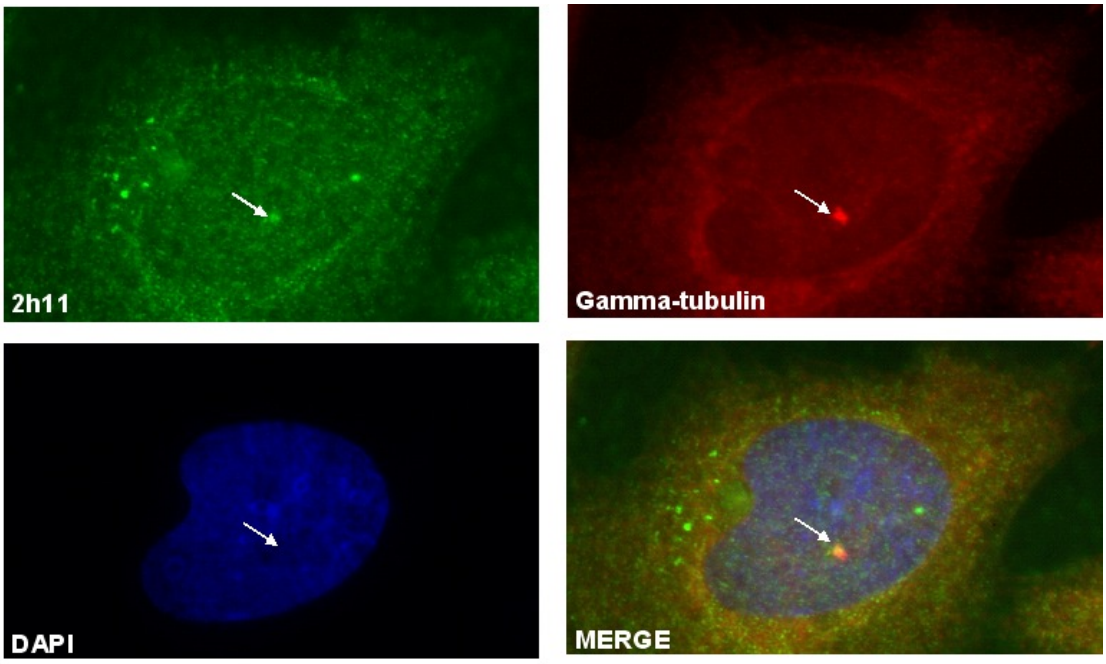


Figure 3-23d. EYFP-Mms2 appears to localize to the centrosomes in SAOS2 cells stably expressing EYFP-Mms2. SAOS2 pEYFP-Mms2 cells were fixed with -20°C methanol for 5 minutes and were then stained with the Mms2/Uev1a-specific mouse 2H11 antibody and the rabbit gamma tubulin antibody. The rabbit gamma tubulin antibody confirms centrosome localization. 2H11 localizes in one of the two centrosomes in this interphase cell.

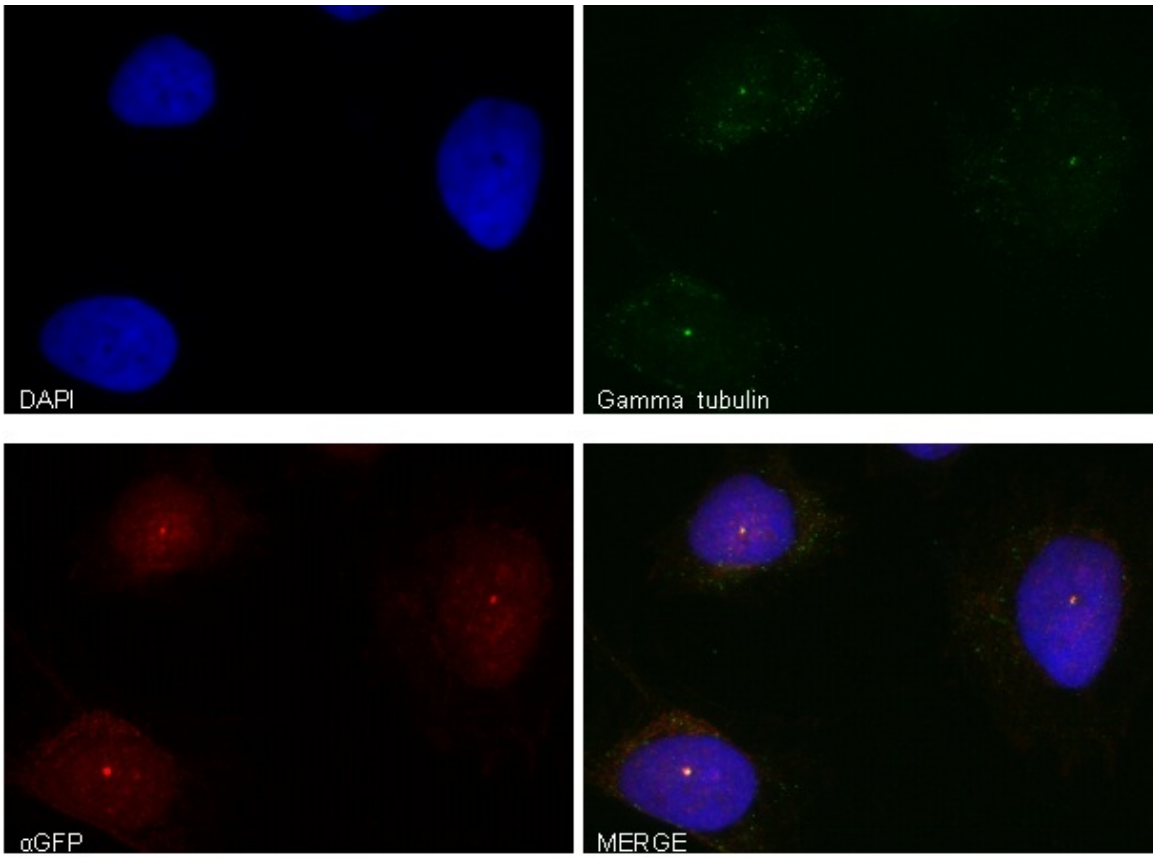


Figure 3-23e. EYFP-Mms2 appears to localize to the centrosomes in SAOS2 cells stably expressing EYFP-Mms2. SAOS2 EYFP-Mms2 cells were fixed simultaneously with 4% formeldahyde and 0.5% NP40 for 15 minutes and were then stained with the rabbit GFP antibody to detect EYFP-Chfr and the mouse gamma tubulin antibody to detect centrosome localization.

CHAPTER 4: DISCUSSION

4.1 Protein interactions indicating involvement of Chfr, Ubc13, and pH3 in mitosis.

Chfr binds to phosphorylated histone H3 (pH3), a protein very important in the G2/M transition. Histones are important for packaging DNA into compact chromosomes, and influence chromosome condensation and decondensation. Phosphorylated histone H3 functions at early prophase and not at interphase. It is a mitotic protein. Chfr binds to pH3, most likely through its FHA domain. The literature is in line with this finding (Chfr-pH3), stating that whenever the FHA domain of Chfr is deleted, this results in a dysfunctional checkpoint, probably due to the inability of Chfr to reduce pH3 levels or mitotic index in a cell population (Scolnick et al., 2000).

A possible explanation for the Chfr disfunction caused by the FHA deletion at a molecular level is that Chfr is no longer able to bind to pH3 and hence cannot influence or remove pH3 from DNA, resulting in pH3 level increase, which indicates chromosome condensation. The literature is in line with this finding, stating that whenever normal Chfr protein levels are low or the FHA domain is deleted, pH3 levels are high (Scolnick et al., 2000).

Since Chfr expression clearly influences the level of this protein, and since the pH3 protein is necessary for chromosome condensation, a Chfr-pH3 interaction is not surprising. The interaction supports the hypothesis that Chfr interacts directly with DNA to rapidly modulate its state of condensation at the interphase-early prophase transition.

There are also some observations that indicate Ubc13 binds to pH3 in mitosis. Ubc13 can make poly-ubiquitin chains with Chfr that are K-63-linked in nature (Bothos et al., 2003). The Chfr-Ubc13, Chfr-pH3, and Ubc13-pH3 interactions support the idea that a) these proteins function in mitosis, because they bind pH3; b) these proteins function together in mitosis; and c) these proteins function together at the early prophase, through K-63-linked or K-48-linked poly-ubiquitination. This bulky polyubiquitination chain could then either generate stearic hindrance to temporarily prevent proper chromosome compaction or prevent access to pH3. Aurora A kinases, for example, would have reduced access to pH3, resulting in a higher likelihood of dephosphorylation of pH3 and decondensation of chromosomes at early prophase, blocking mitotic progression.

4.2 Chfr localization to decondensed or decondensing chromatin indicating mitotic function.

Furthermore, an over-expressed Chfr protein (EGFP-Chfr) in U2OS cells has a tendency to accumulate at higher concentrations in particular cellular structures, most notably the nucleus at interphase and telophase. Interestingly, these are the two cell cycle stages where chromatin is either kept in a decondensed state, or a decondensing state, respectively. This accumulating concentration of the Chfr protein within the vicinity of the decondensed or decondensing DNA indicates a decondensing role for Chfr.

There are also some observations that indicate Ubc13 localizing to mitotic chromosomes, also including telophase. Such visual localizations are significant

because they reflect, support, and help to elucidate some of the earlier findings about the molecular mechanism of Chfr. For example, Scolnick and Halazonetis (2000) showed how Chfr and Ubc13 could function together using the ubiquitination mechanism to delay mitosis.

4.3 Chfr, Ubc13, and Mms2/Uev1a localization to mitotic structures.

Chfr, Ubc13, and Mms2/Uev1a localize to the centrosomes. Localization of Chfr, Ubc13, and Mms2/Uev1a to the centrosomes indicates that these proteins are involved in centrosome maturation, separation, and/or spindle formation. Aurora A and Plk1, two mitosis promoting proteins, also have been found to localize to the centrosome structures, and are important for proper cell cycle progression. Our results indicate that Chfr, Ubc13, and Mms2/Uev1a localize to these structures, which indicates that they may possibly function together at these sites to send signals to mitotic regulators, such as Aurora A or Plk1. Ubc13 presence at the centrosomes implies a potential K-63-linked poly-ubiquitinating function at these sites, though a K-48-linked function is not excluded. Ubc13 and Mms2 have also been found in the mitotic spindle of some cells.

Summers et al. (2005) shows how Chfr expression influences Aurora A. Chfr expression in cells appears to prevent the accumulation of active Aurora A at the interphase centrosomes. Active Aurora A (Aurora pT288) is only present in cells that are in mitosis and have thus overcome the Chfr checkpoint. Perhaps Chfr functions here by ubiquitinating Aurora A for destruction by K-48-linked poly-

ubiquitination at interphase, and at late prophase once cells squeeze through the G2/M checkpoint activates Aurora A by K-63 linked poly-ubiquitination using the Ubc13-Mms2 enzyme. Cells can then ensure that their centrosomes mature properly, that the mitotic spindle is properly formed, and that mitotic progress occurs securely at the spindle checkpoint. Accurate cell division can in this way be properly carried out (Summers et al., 2005).

Toland et al. (2003) further helps to confirm the localization of Ubc13 to the centrosomes at mitosis. They do not mention Chfr. However, they do mention that when Aurora A is mutated, Ubc13 does not colocalize to the centrosomes with it. This indicates that Aurora A may recruit Ubc13 into the centrosomes during mitosis to carry out a specific function. This report together with our results indicates that Ubc13 functions with Chfr to modulate Aurora A function. Ubc13 presence implies the possibility of K-63-linked poly-ubiquitination (Toland et al., 2003). The Chfr checkpoint, however, most likely uses K-48-linked poly-ubiquitin chains to degrade or inactivate Aurora A, preventing centrosome maturation and spindle formation during microtubule damage. Only after microtubule stress is removed can the cell progress through mitosis. At this point Ubc13 could use K-63-linked poly-ubiquitin chains at the centrosomes to activate important mitotic regulators, allowing for centrosome maturation, spindle formation, and/or proper progression through mitosis.

Thus, a molecular mechanism can be envisioned here, whereby Chfr localizes to centrosomes with Ubc13-Mms2, which would be recruited by Aurora A (Toland et al. 2003). This complex could then generate K-48 linked signals to reduce Aurora A,

Plk1, or alpha-tubulin levels (Privette et al., 2008b), or K63-linked signals to influence protein location or protein-protein interactions at the centrosomes and microtubule location.

4.4 Chfr level and modification at the G2/M mitotic entry gate.

Kim et al. (2011) shows a clear correlation between Chfr protein level and pH3 protein level, as cells enter mitosis and progress through mitosis. This group clearly shows that when Chfr expression decreases, pH3 levels increase, which indicates increased entry into mitosis. They also show that such fluctuations in Chfr and pH3 level may be due to a degradative ubiquitin signal, with Chfr reducing itself in level when not necessary.

4.4.1 The two molecular states of Chfr at G2/M transition. Looking at the state of Chfr in cell populations that are at a particular time point, can give a glimpse into how the protein could function at a molecular level. Our data indicates that (a) there are two molecular states of Chfr: Chfr and Chfr-Ub; (b) Chfr is predominant at LP, whereas, Chfr–Ub is predominant at INT/EP; (c) Chfr looses its ubiquitin signal after cells pass through the INT/EP transition; (d) Chfr protein levels clearly increase at LP in response to extensive mitotic stress, after passing through the INT/EP stage; (e) Chfr, Ubc13, and Mms2/Uev1 levels increase at INT/EP indicating their importance at the G2/M checkpoint gate; and f) the presence of high Ubc13 and Mms2/Uev1 levels indicates the possibility of K63-linked poly-ubiquitination at that particular time point.

4.4.2 Association of the Chfr modification with chromosome condensation.

Chfr-Ub is associated with reduced pH3 levels and deubiquitinated Chfr with increased pH3 levels in a cell population. The ubiquitin signal could be either K63-linked or K48-linked as Ubc13 can generate both types of poly-ubiquitin chains. Although the data obtained in this study indicate a potential K63-linked poly-ubiquitin modification, this does not exclude a degradative signal, particularly since low Chfr level is correlated with high ubiquitination.

Here a model could be envisioned whereby the E3 ligase functions together with Ubc13 and Mms2 at the INT/EP checkpoint gate to decondense DNA. The ubiquitin signal could be either K-63-linked or K-48-linked in nature. Chfr-Ub could bind directly to pH3 at the INT/EP stage, and its auto-ubiquitin signal provide temporary stearic hindrance to interfere with chromosome condensation**.** Following the temporary delay at the INT/EP transition due to microtubule stress, Chfr could then destruct to remove stearic hindrance and allow chromosome condensation, using its temporary ubiquitin signal. Stearic hindrance would be reduced in this way. Upon passage through INT/EP, Chfr clearly looses its ubiquitin signal**.** As a result, Chfr increases in level upon entry into LP; and this could serve as a further control to prevent progression under suspicious conditions**.** Under normal conditions, Chfr would be expected to auto-ubiquitinate and eventually destruct upon entry into mitosis, resulting in an increase in pH3 levels**.** Temporary auto-ubiquitination could in this way provide a decondensation model where Chfr bind pH3, auto-ubiquitinates self, and decondenses DNA.

4.4.3 Auto-ubiquitination versus ubiquitination of substrate proteins. Furthermore, Chfr could bind to pH3 and generate a bulky K-48/K-63 linked poly-ubiquitin stress signal to prevent access to important mitotic kinases that phosphorylate and activate H3. Chfr could poly-ubiquitinate either itself, pH3 or the mitotic kinases themselves to generate stearic hindrance. This could interfere with protein level, protein-protein interactions, protein function, or protein localization.

Chfr could influence localization of Aurora A to the centrosomes (Summers et al., 2005) by using a K63-linked poly-ubiquitin signal. When Chfr is over-expressed, for example, Aurora A cannot be activated at interphase centrosomes (Summers et al., 2005). It is only activated later on in mitosis. Perhaps Chfr transfers a bulky K63-linked ubiquitin signal onto the Aurora A protein that disables Aurora A from localizing to the centrosomes. Alternatively, a K48-linked poly-ubiquitin signal could degrade Aurora A at the interphase centrosomes (Yu et al., 2005; Summers et al., 2005).

Chfr could also bind to histone H3 at INT/EP, generate a bulky K63-linked poly-ubiquitin signal, and prevent access of Aurora B to histone H3 at this stage. Unable to access histone H3, Aurora B would be prevented from phosphorylating histone H3 on Serine 10, preventing mitotic entry. Aurora B can localize to the kinetochores at G2 (Summers et al., 2005). Kinetochores are specialized regions of DNA that contain specialized histones such as histone H3 variant CenH3, and are DNA regions where microtubules eventually attach at the spindle checkpoint.

Alternatively, as far as centrosomes are concerned, Chfr could degrade alpha-tubulin; Aurora A, and Plk1 would then be expected for targeted destruction by Chfr at the centrosomes. It could degrade Plk1 by K48-linked poly-ubiquitination, activate CyclinB1-Cdc2 and promote mitotic entry.

4.4.4 Influence of nocodazole on Chfr modification. Furthermore, nocodazole which is a form of microtubule stress, affects the observed Chfr modification. As cells are exposed to nocodazole for a longer time period, Chfr protein levels increase but the modification on this protein decreases. Since the modification decreases upon nocodazole exposure, this indicates that nocodazole exposure probably results in Chfr deubiquitination. Cells that are not exposed to nocodazole have the most modified Chfr, and the least Chfr protein, whereas cells that are exposed for longer periods have less modification and more protein. This indicates that Chfr may auto-ubiquitinate itself for destruction when not necessary.

4.5 Chfr and its relation to chromatin.

As far as chromosomes are concerned, the poly-ubiquitin chains of Chfr could possibly serve to change the conformation of the chromatin and increase stearic hindrance at the early prophase stage of mitosis. This is in line with the observation that a) Chfr binds to pH3, which is an early mitotic protein; b) Ubc13 binds pH3; c) Chfr binds to Ubc13, which is a ubiquitin-conjugating enzyme d) Chfr auto-ubiquitinates itself; e) the ubiquitin modification increases, as pH3 levels decrease in nocodazole presence; and f) Chfr-Ub is associated with INT/EP.

Nocodazole exposure can regulate the amount of Chfr-Ub in a cell population, and the amount of phosphorylated histone H3 in a cell population. Mitotic chromosomes are rich in phosphorylated histone H3. The more nocodazole, the less polyubiquitinated Chfr in a cell population, the more Chfr there is in the cell population, and the more phosphorylated histone H3 there is in nocodazole-exposed cells. In nocodazole presence, the Chfr-pH3 interaction appears to be lost, and the amount of poly-ubiquitin chains on Chfr is also lost: as nocodazole increases, ubiquitinated Chfr decreases. This could reduce steric hindrance potentially generated by the ubiquitin chains, leading to condensation. In other words, Chfr cannot decondense the chromosomes because the Chfr signal is gone. In the presence of nocodazole, Chfr can no longer bind to pH3, so it cannot affect DNA structure, which is most likely why pH3 levels rise as nocodazole exposure increases**.**

In the absence of nocodazole, ubiquitinated Chfr could bind histone H3 through the FHA domain (Stavridi et al., 2002). Ubiquitinated Chfr is correlated with decreased amounts of pH3 (decreased chromosome condensation). -The bulky ubiquitin signal on the Chfr protein bound to pH3 could serve to decondense DNA at interphase and, when conditions are favorable, lead to the destruction of the Chfr protein to allow entry into mitosis. **Figure 4-1** summarizes the discussion. Since pH3 symbolizes chromosome condensation and mitosis, the correlation between increased Chfr ubiquitination and decreased chromosome condensation (decreased pH3 levels) in nocodazole presence, indicates a mitosis-preventing role for Chfr-Ub.

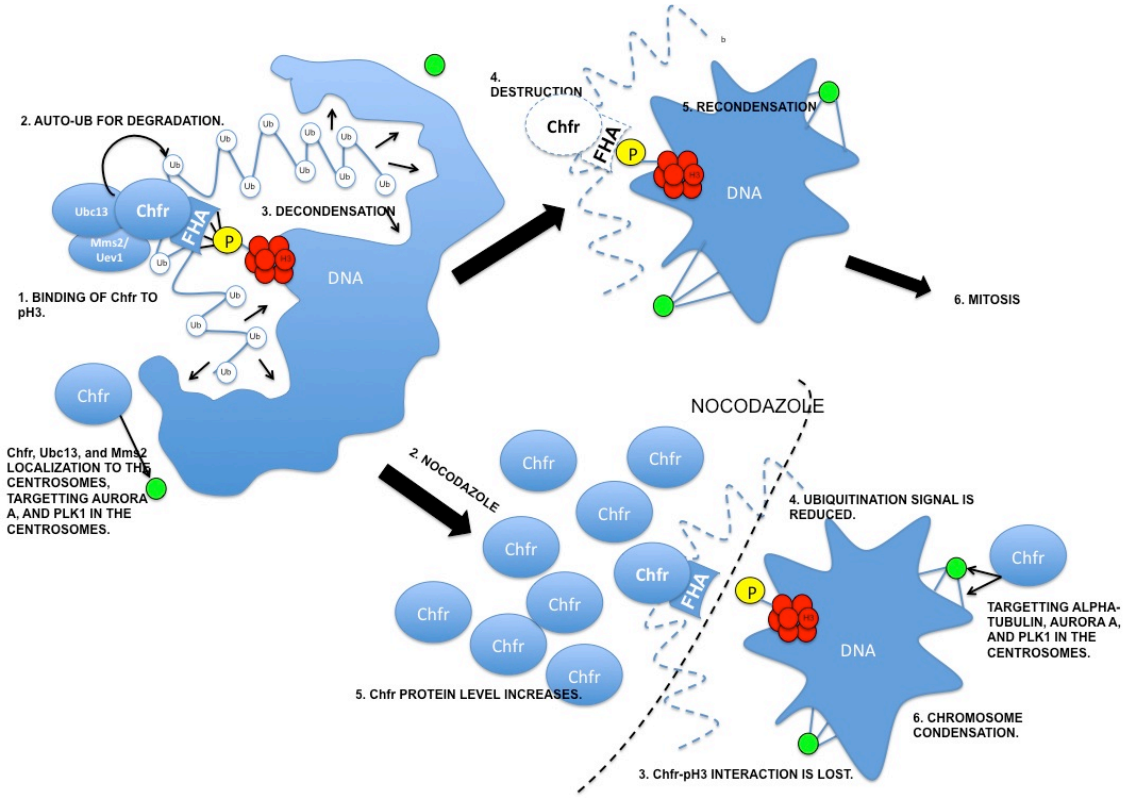


Figure 4-1. A possible molecular mechanism by which Chfr, Ubc13, and Mms2 could function together to promote accurate mitosis. The evidence presented in the report indicates that Chfr, Ubc13, and Mms2 localize to the centrosomes during mitosis. The Chfr-Ubc13-Mms2 protein complex could possibly alter the level or behavior of important cell cycle promoting proteins in the centrosomes and in the mitotic spindle to halt mitosis. Aside from the centrosome localization, the Chfr-Ubc13-Mms2/Uev1 complex could also function in chromosome decondensation to prevent mitotic progression. (1) Chfr binds to pH3 via its FHA domain at interphase-early prophase. (2) The Chfr-Ubc13-Mms2 complex auto-ubiquitinates itself at interphase-early prophase. This signal could be either K48 or K63-linked. (3) The chromatin is kept in a decondensed state in interphase and in telophase, and the bulky ubiquitin chain interferes with chromosome condensation. (4) The Chfr protein level is reduced through degradation, which is the reason why (5) chromosomes condense and (6) mitosis occurs.

Upon extensive nocodazole treatment (2), the Chfr-pH3 interaction is lost (3), the ubiquitin signal is reduced (4), and Chfr protein level increases in late

prophase to counteract the mitotic stress (5). Chromosomes condense (6) because Chfr can no longer bind to pH3 on chromosomes and it can no longer use the bulky ubiquitin signal to interfere with chromosome condensation. Upon temporary nocodazole exposure Chfr could target Plk1, Aurora A, and acetylated-alpha-tubulin for destruction at the centrosomes and at the mitotic spindle.

CHAPTER 5: CONCLUSIONS

5.1 Localization of Chfr, Ubc13, and Mms2 to mitotic structures indicates their function in mitosis.

5.1.1 Localization to the centrosomes. Immunocytochemistry indicates that Chfr, Ubc13, and Mms2 localize to the centrosomes in interphase and in mitosis. Centrosomes are microtubule-organizing centres in cells, and their proper growth, maturation, duplication, and separation at interphase and early prophase is necessary for normal mitosis. Localization of Chfr, Ubc13, and Mms2 to these structures, thus, indicates their function. Colocalization of Chfr and Ubc13 to the structures indicates that they function together. High levels of Chfr-Ub, Ubc13, and Mms2 at the interphase-early prophase stage, and not at the late prophase stage, further helps to support the immunocytochemistry results that the proteins function together.

Aurora A and Plk1, two mitosis promoting proteins, also localize to the centrosomes (Privette et al., 2008b; Toland et al., 2003; Summers et al., 2005). Based on the results and on the literature, a molecular mechanism can be envisioned whereby Chfr, Ubc13, and Mms2/Uev1 reduces the level of Aurora A or Plk1 in the centrosomes at interphase through K48-linked poly-ubiquitination. Acetylated alpha-tubulin in the mitotic spindle apparatus could also be targeted for destruction. Auto-ubiquitination of Chfr would be useful here in modulating the level of Chfr: auto-ubiquitinating itself for destruction when not necessary and

increasing itself in level when exposed to nocodazole. Once the mitotic stress is removed Ubc13-Mms2 could then generate K63-linked poly-ubiquitin chains to alter protein function of Aurora A, or Plk1. It could also stabilize certain proteins such as acetylated-alpha tubulin. The hypothesis was that Chfr, Ubc13, and Mms2 are involved in mitosis, and their localization to mitotic structures indicates that the results agree with this hypothesis.

5.1.2 Localization to the mitotic spindle. Both Ubc13 and Mms2 have been found to localize to the centrosomes and to the mitotic spindle of some cells. This indicates that the two proteins are involved in mitosis. The spindle checkpoint occurs at the metaphase-to-anaphase transition to ensure that all microtubules are properly attached to the kinetochores before anaphase can occur. Chfr has been shown to bind to alpha-tubulin, and to ubiquitinate this protein for destruction (Privette et al., 2008b). Perhaps the bulky auto-ubiquitin signal on Chfr, which binds to alpha-tubulin, prevents binding to kinetochores. Lack of proper attachment between kinetochores and microtubules could then further alarm the spindle checkpoint into action.

5.1.3 Localization to the DNA. Furthermore, immunocytochemistry indicates that Chfr localizes to decondensed (interphase) or decondensing (telophase) chromatin, which gives a visual representation of its function at the vulnerable G2/M entry gate. At interphase the nucleus is full of Chfr, and chromatin remains decondensed.

After cells enter mitosis, Chfr no longer localizes to DNA, dispersing throughout the cytoplasm. Reaccumulation of Chfr in telophase confirms the decondensing function of Chfr in interphase, as telophase is a stage at which DNA starts to decondense. Chfr localization at the interphase stage and at the telophase stage supports the idea that Chfr functions at the beginning and at the end of mitosis to decondense DNA, which supports the hypothesis that this protein is involved in mitosis, and that its function is of a decondensing nature.

Ubc13 also localizes to DNA. It localizes to early prophase chromosomes, throughout mitosis, and also to telophase chromatin. Both Ubc13, and Chfr, thus, associate with DNA, which is in line with the literature that states that Ubc13 and Chfr function at the nucleus to guard mitotic progression.

5.2. High Ubc13 levels in mitosis and Ubc13 interaction with mitotic chromosomes indicates a mitotic function for Ubc13.

Coimunoprecipitation indicates that Chfr and Ubc13 may interact with phosphorylated histone H3. pH3 is a very important indicator of mitosis, as this histone becomes phosphorylated very early on in mitosis, just as chromatin is beginning to condense. It is also very useful in distinguishing early prophase cells from interphase cells and late prophase cells. It is at the early prophase stage of mitosis, where the Chfr protein has been shown to exert its effects. The Chfr-pH3 and Ubc13-pH3 interactions, therefore, support the hypothesis that Chfr and Ubc13 play a role in mitosis, and especially in early mitosis. In addition, Ubc13

immunocytochemistry shows that Ubc13 may be able to bind mitotic chromosomes, which helps to verify these coimmunoprecipitation results. Synchronization by the 2xThymidine-1xRO3306 block and Western blot analysis further confirm Ubc13 involvement in mitosis because these two techniques show that Ubc13 levels increase as synchronized cells enter mitosis following the G2/M block. In summary, the results indicate Ubc13 increases in mitosis, binds mitotic chromosomes, and binds to phosphorylated histone H3. These results indicate a role for Ubc13 in regulating the state of chromosome condensation and decondensation. The level of Ubc13 and Mms2 is also increased at the interphase-early prophase time point, and this further supports the idea that Ubc13 could function at that particular point with Chfr, during the Chfr checkpoint.

5.3. Modification of Chfr at the G2/M transition is correlated with reduced Chfr levels and chromosome condensation.

Mitotic shake off and coimmunoprecipitation indicates that Chfr is ubiquitinated at the interphase-early prophase stage of mitosis. This signal could be K63-linked or K48-linked, but is most likely degradative in nature as Chfr protein levels have been shown to decrease upon mitotic entry to allow cells into mitosis.

Our results indicate that Chfr is noticeably ubiquitinated at the interphase-early prophase transition. The ubiquitin signal is clearly lost as cells pass the LP stage of mitosis. Also, in the presence of nocodazole, Chfr protein levels clearly increase to counteract the mitotic stress. High Ubc13 and Mms2 protein levels

coincide with high levels of Chfr-Ub, indicating that they function together at this time point. The Chfr-pH3, Ubc13-pH3 and Chfr-Ubc13 interaction helps to support the conclusion that Chfr, Ubc13, and Mms2 function together at early prophase. The ubiquitin signal is expected to regulate mitotic entry into mitosis. When the ability of Chfr to auto-ubiquitinate itself is gone, Chfr expressing cells enter mitosis more often than those expressing normal Chfr. The polyubiquitin signal could be K63-linked or K48 linked. Perhaps Chfr uses the polyubiquitin signal to temporarily promote a decondensed state of chromatin at interphase-early prophase, using the bulky signal as a form of stearic hindrance at the histone site.

5.4 The Chfr, Ubc13, and Mms2 protein complex could function through a self-ubiquitination-decondensation-destruction-recondensation mechanism.

In summary, the data indicates that Chfr and Ubc13 both interact with phosphorylated-histone H3, which is a very important component of condensing chromatin. Chfr and Ubc13 also bind to one another. Based on our research findings, it is possible that Chfr and Ubc13 could function together to decondense or keep DNA in a decondensed state by binding to this mitotic histone, using its auto-ubiquitinating signal. A ubiquitin molecule is 76 amino acids in size and histone H3 is 136 amino acids in size. So a chain of ubiquitin attached to Chfr, which binds pH3, could be considered bulky and could, in theory, interfere with compact chromatin condensation. In other words, auto-ubiquitination of Chfr could lead to dynamic decondensation, and then destruction of the Chfr-Ub protein (and the bulky ubiquitin signal), leading to recondensation and mitosis. When nocodazole is

added to such a situation, Chfr levels increase to counteract the mitotic stress. However, this also results in loss of the Chfr-pH3 interaction, and loss of Chfr-Ub, indicating loss of bulky ubiquitin chains near the chromatin, resulting in chromosome condensation and forced entry into mitosis. Chfr could also localize to centrosomes and the mitotic spindle upon temporary nocodazole exposure to reduce acetylated alpha-tubulin, Plk1, and Aurora A. **Figure 4-1** explains the molecular mechanism by which Chfr, Ubc13, and Mms2 could function together to promote accurate mitosis.

REFERENCES

Bothos J, Summers MK, Venere M, Scolnick DM, Halazonetis TD. (2003). The Chfr mitotic checkpoint protein functions with Ubc13-Mms2 to form Lys63-linked polyubiquitin chains. *Oncogene*;22(46):7101-7107.

Cano E, Hazzalin C, and Mahadevan L. (1994). Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. *Molecular Cell Biology*;14(11):7352-7362.

Chaturvedi P, Sudakin V, Bobiak ML et al. (2002). Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. *Cancer Res*;62(6):1797-1801.

Cooper S, Iyer G, Tarquini M, and Bissett P. (2006). Nocodazole does not synchronize cells: implications for cell-cycle control and whole-culture synchronization. *Cell and Tissue Research*;24(2):237-242.

Erson A.E. and Petty E.M**.** (2004). CHFR-associated early G2/M checkpoint defects in breast cancer cells. *Molecular Carcinogenesis* 39:26-33.

Fukuda T, Kondo Y, Nakagama H. (2008). The anti-proliferative effects of the CHFR depend on the forkhead associated domain, but not E3 ligase activity mediated by ring finger domain. *PLoS ONE*;3(3):e1776.

Hirota T, Kunitoku N, Sasayama T, Marumoto T, Zhang D, Nitta M, Hatakeyama K, Saya H. (2003). Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell*.;114(5):585-98.

Kang D, Chen J, Wong J, Fang G. (2002). The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J Cell Biol*;156(2):249-259.

Kim JS, Park YY, Park SY, Cho H, Kang D, and Cho H. (2011). The auto-ubiquitylation of E3 ubiquitin protein ligase Chfr at G2 phase is required for accumulation of polo-like kinase 1 and mitotic entry in mammalian cells. *J Biol Chem*. 286(35):30615-23.

Matsusaka T, Pines J. (2004). Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. *J Cell Biol*;166(4):507-516.

Newton K, Matsumoto M, Wertz I, Kirkpatrick D, Lill J, Tan J, Dugger D, Gordon N, Sidhu S, Fellouse F, Komuves L, French D, Ferrando R, Lam C, Compaan D, Yu C, Bosanac I, Hymowitz S, Kelley R, and Dixit V. (2008). Ubiquitin Chani

Editing Revealed by Polyubiquitin Linkage-Specific Antibodies. Cell; 134: 668-678.

Ogi K, Toyota M, Mita H, Satoh A, Kashima L, Sasaki Y, Suzuki H, Akino K, Nishikawa N, Noguchi, M, Shinomura Y, Imai K, Hiratsuka H, Tokino T. (2005). Small interfering RNA-induced CHFR silencing sensitizes oral squamous cell cancer cells to microtubule inhibitors. *Cancer Cell Biology*;4(7):773-

Oh YM , Kwon YE , Kim LM, Bae SJ, Lee BK, Yoo SJ, Chung CH, Deshaies RJ & Seol JH. (2009). Chfr is linked to tumor metastasis through the downregulation of HDAC. *Nature Cell Biology.*;11:295-302.

Privette LM and Petty EM. (2008a). CHFR: A novel mitotic checkpoint and regulator of tumorgenesis.Translational Oncology;1(2): 57-64.

Privette L, Weier J, Nguyen H, Yu X, and Petty E. (2008b). Loss of CHFR in human mammary epithelial cells causes genomic instability by disrupting the mitotic spindle assembly checkpoint. *Neoplasia*;10(7):643-652.

Scolnick DM, Halazonetis TD. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature*;406(6794):430-435.

Stavridi ES, Huyen Y, Loreto IR et al. (2002). Crystal structure of the FHA domain of the Chfr mitotic checkpoint protein and its complex with tungstate. *Structure*;10(7):891-899.

Summers MK, Bothos J, Halazonetis TD. (2005). The CHFR mitotic checkpoint protein delays cell cycle progression by excluding Cyclin B1 from the nucleus. *Oncogene* ;24(16):2589-2598.

Takenaka K, Moriguchi T, Nishida E. (1998). Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science*;280:599-602.

Ewart-Toland A, Briassouli P, P de Koning J, Mao J, Yuan J, Chan F, MacCarthy-Morrough L, Ponder B, Nagase H, Burn J, Ball S, Almeida M, Linardopoulos S, Balmain A (2003). Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nature Genetics.* 34(4):403-412.

Vassilev L, Tovar C, Chen S, Knezevic D, Zhao X, Sun H, Heimbrook D, and Chen L. (2006). Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc Natl Sci USA* 103(28):10660-10665.

Wang C, Deng L, Hong M, Akkaraju G, Inoue J, & Chen ZJ. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*;412:346-351.

Yu X, Minter-Dykhouse K, Malureanu L et al. (2005). Chfr is required for tumor suppression and Aurora A regulation. *Nat Genet*. 37(4):401-406.

APPENDIX (Antibodies, solutions, buffers, media, chemicals, reagents):

Dithiothreitol (DTT)
BP 172-5
Fisher scientific

Deoxycholic Acid
D-6750
SIGMA

Igepal CA-630 (Nonindet P40)
SIGMA-ALDRICH
13021-100 ml

Iodoacetamide, SigmaUltra
I1149
SIGMA

***N*-Ethylmaleimide**
E1271-5G
SIGMA-ALDRICH

N,N,N′,N′-Tetramethylethylene-diamine, for electrophoresis, approximately 99%
T9281-25 ml
SIGMA

Acrylamide/Bis-acrylamide
A6050-100 ml
SIGMA

Thimerosal, SigmaUltra
T8784-1G-25 ml
SIGMA

Lipofectamine RNAiMAX Reagent
13778-030
Invitrogen

OPTI-MEM®1
31985-062
GIBCO Invitrogen

R03306
217-699
CALBIOCHEM

Monoclonal Anti-β Actin antibody
Produced in mouse
Clone AC-74, ascites fluid
SIGMA A5316 - 0.5 ml

Anti-phospho-histone H3 antibody
(Ser10) Mitosis Marker
06-570
MILLIPORE

GFP Antibody
sc-8334
rabbit polyclonal IgG
Santa Cruz Biotechnology

2H11 ascites
α-hMMS2
Feb 19/01

Anti-Ubiquitin, Lys 63-Specific antibody
Clone Apu3 (rabbit monoclonal)
05-1308
MILLIPORE

Mouse Gamma Tubulin antibody
05-565
UPSTATE

Anti-Myc Tag antibody
clone 4A6 agarose conjugate
16-219
Millipore

Anti-Myc Tag antibody
rabbit polyclonal IgG
06-549
UPSTATE

Goat anti-mouse IgG HRP conjugate antibody
UPSTATE
12-349

goat anti-rabbit IgG HRP conjugate antibody
sc-2004
HRP conjugated
Santa-Cruz Biotechnology

goat anti-mouse IgG HRP conjugate antibody
12-349
UPSTATE

Anti-Xpress antibody
46-0528
Invitrogen

Chfr (H-300)
sc-28263
Rabbit polyclonal IgG
Santa Cruz Biotechnology

0.05% Trypsin-EDTA
25300-062
Invitrogen GIBCO

FBS
10437
GIBCO Invitrogen

Bio-Rad Protein Assay
BIORAD
500-0006

Acrylamide/Bis-acrylamide, 37:1
SIGA-ALDRICH
A6050-100 ml

Iodoacetamide
Sigma Ultra
SIGMA
I1149-5 g

N-Ethylmaleimide
E1271-5 g
SIGMA-ALDRICH

Methanol
A412-4
Fisher scientific

Lysis Solution:
200 µl of phosphate buffer (0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 50 ml ddH_2O)
54.8 µl 5M NaCl
20 µl of phosphate inhibitory cocktail
20 µl 1 M NEM in DMSO
40 µl 10% SDS
40 µl 10% deoxycholate
1625 ml water

IP diluent:
1000 µl of phosphate buffer
274 µl 5 M NaCl
100 µl phosphate inhibitory cocktail
100 µl 1 M NEM dissolved in DMSO
8526 ml of water

Elution buffer:
125 µl loading buffer
25 µl phosphate buffer
50 µl 10% SDS (1% final)
5 µl 1M DTT
295 µl autoclaved ddH_2O
6 µl 0.5 M iodoacetamide per tube